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Oral Use of Penicillin in Treatment of Experimental *Erysipelothrix rhusiopathiae* Infection in Mice.

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The development of an effective treatment for swine erysipelas becomes of considerable importance because of the increasing prevalence of this disease in the United States and in foreign countries. The present study was therefore undertaken to evaluate the use of penicillin in the control of experimental infection. Heilman and Herrell[1] have reported successful treatment of infected mice by frequent injections of penicillin, totaling 1000 units per day per mouse, over a 7-day period. Since simplicity of treatment often becomes a prime factor in the field of animal disease control, we were particularly interested in the possibility of oral administration rather than the injection method.

Procedures. *Mice:* Test animals throughout were 16 to 18 g mice. For the major portion of tests, Rockland female mice were used.† At one stage it was necessary to substitute a Dilute Brown Agouti, tumor-susceptible strain due to scarcity of the Rocklands. Comparative tests using the 2 strains showed no difference in response.

Penicillin-in-feed: In early trials penicillin was suspended in corn oil in a concentration of 1000 units per cc of oil. This was then mixed with a cereal type feed to give a final concentration of either 100 or 1000 units of penicillin per gram of feed. In later tests, the dried penicillin was ground with dried feed in varying concentrations and fed with no protective coating of oil. Test mice were placed in individual cages and given weighed amounts of penicillin-containing food. After 24 hours, animals were challenged with an injection of 10 MLD of test culture. Feeding with treated material was continued for the

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* American Cyanamid Research Laboratories, Stamford, Connecticut.

[1] Heilman, F. R., and Herrell, W. E., *Proc. Staff Meetings of the Mayo Clinic*, 1944, **19**, 340.

† Obtained from Rockland Farms, New City, N.Y.

TABLE I.
Effectiveness of Penicillin in Feed.

Method of administration	10 units/g	100 units/g	1000 units/g	2500 units/g
Oil	0/10*	0/10	0/10	0/10
Dry	0/10	0/10	10/10	10/10

* Survivals/mice injected.

full time of the experiment. A like number of animals were fed a normal diet and served as control animals.

Penicillin-Drinking Water: Penicillin was diluted with tap water in concentrations ranging from 10 to 1000 units per cc of water. Fifty cc amounts of final dilution were supplied to each cage of 5 mice. This amount was enough for 48 hours in most instances. The penicillin-water was replaced every 48 hours with fresh material, regardless of any amount which might remain. In original trials, animals were given the treated drinking water for 24 hours to develop a blood level prior to challenging. In later experiments the challenge dose and beginning of treatment were simultaneous. In final trials, the challenge took place 24 or 48 hours prior to the beginning of treatment. The length of time for continuance of treatment varied from 24 hours to 10 days—the duration of tests.

Culture: The test culture used throughout was *Erysipelothrix rhusiopathiæ*, Lederle No. 358. This strain is used routinely for protection tests against anti-swine erysipelas serum and is of extremely high virulence for mice. Subcutaneous injection of 0.5 cc of a 10^{-8} dilution of culture will consistently kill 100% of unprotected mice, and a similar injection of a 10^{-9} dilution usually kills at least 2 out of 3 mice.

Results. Effectiveness of penicillin-in-feed: The oil-coated penicillin appeared to be very unpalatable and was eaten in smaller amounts than the control feed. For this reason, the mice failed to develop a protective blood level and all animals died within 4 days from the date of challenge. When the dried material was ground with the feed in a concentration of 1000 units per gram, or more, a satisfactory level was obtained and 100% protection was afforded, while all control animals died within 4 days of the date of challenge. (Table I shows sample experiment.)

Effectiveness of penicillin-in-water: Addition of 1000 units of penicillin per cc of water provided complete protection when begun in advance of or simultaneously with the injection of the challenge dose. When the concentration was cut to 500 units per cc, the protective rate dropped to 20% of the test animals. Concentrations lower than 500 units per cc were of no protective value even when supplied for 48 hours prior to the date of challenge. (Table II.)

Tests were then carried out to determine whether or not the 1000-unit concentration would be effective against more than the 10 MLD challenge dose. Injections were made from serial dilutions prepared to give 100, 1000, and 10,000 MLD and treatment was begun simultaneously. Complete protection was provided against even the highest number of MLD used for challenging.

When challenge injections were made prior to the beginning of treatment, results were variable. Treatment begun 24 hours after challenge in some instances gave almost complete protection, while in duplicate trials only 20% survival resulted. After 48 hours higher

TABLE II.
Effectiveness of Penicillin in Drinking Water.

Treatment begun	10 units/cc	100 units/cc	500 units/cc	1000 units/cc	5000 units/cc	10,000 units/cc
24 hr before challenge	0/10	0/10	2/10	10/10	10/10	10/10
Simultaneously with challenge	—	—	0/10	10/10	10/10	10/10
24 hr after challenge	—	—	—	4/10	5/10	5/10
48 hr after challenge	—	—	—	0/10	0/10	0/10

dosages, even up to 10,000 units per cc, were of no value and all animals died.

Discussion. The evidence presented here indicates that penicillin, with no protective coating or buffering materials, may be successfully administered to mice by mouth and, provided that sufficiently large quantities are given, protection is afforded against *E. rhusiopathiæ*. Since 50 cc of water containing 1000 units/cc was consumed by 5 mice in 48 hours, the effective average intake per mouse was 5000 units/day. This is approximately 5 times the amount used by Heilman and Herrell[1] in injecting the material. Penicillin is effective in the treatment of *Erysipelothrix rhusiopathiæ* infection if given early enough after exposure. Our results on this point are in accord with the findings of Heilman and Herrell[1] who showed protective action by the injection method of administration when begun 21 to 24 hours after challenge. Under our experimental conditions the effective time interval was 24 hours or less, but in field conditions institution of therapy at such an early stage of infection may be unattainable. It would seem, therefore, that penicillin may be of considerable value only in the control of the outbreak of this infection in domestic animals. The ease of oral administration makes its use feasible on a far greater scale than would be possible where injections are necessary.

Summary. Penicillin is shown to be of value in the protection of mice against experimental *Erysipelothrix rhusiopathiæ* infection.

Oral administration is successful in the treatment of mice even without any protective action from oil coating, or buffers, provided it is instituted within 24 hours after exposure. The use of penicillin in drinking water is found to be as effective as is its addition to the feed. Oral administration compares favorably with parenteral injection although larger amounts are required.

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Preparation of Human Typing Sera by Iso-immunization of Human Donors with Group Specific Substances.

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Typing sera with extremely high agglutination titers have been prepared by Witebsky and his coworkers[1] in human donors following the intravenous injection of solutions containing minute amounts of group A and B specific substances. Wiener,[2] employing intramuscular injections of autoclaved saliva from group A and B individuals (secretors) instead of isolated group specific substances, confirmed Witebsky's observations. Pillemer *et al.*[3] have prepared as by-products of plasma fraction, concentrated isohemagglutinin globulins that were characterized not only by their high titer, but by their high avidity as well, *i.e.*, by the speed with which the serum agglutinated red cells. To the best of our knowledge, an investigation of the change in the avidity of typing sera following isoimmunization of human donors with group specific substances has not appeared in the literature. In the course of studies on blood group specific substances, we have had the opportunity to make such observations.

Nine subjects, 3 belonging to each of the following groups, O, A, and B, received intravenously 0.5 cc of a solution of group A and B

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[1] Witebsky, E., Klendshoj, N. C., and McNeil, C., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 167.

[2] Wiener, A. S., Soble, R., and Polivka, H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 310.

[3] Pillemer, L., Oncley, J. L., Melin, M., Elliot, J., and Hutchison, M. C., *J. Clin. Invest.*, 1944, **23**, 550.

TABLE I.
Avidities and Titers of Blood Grouping Sera Prepared by Immunization of Donors with Group Specific Substances.

Serum preparation		Avidity (seconds)				Agglutination titer			
		Group ''B'' cells		Group ''A'' cells†		Group ''B'' cells		Group ''A'' cells†	
		*	**	*	**	*	**	*	**
Group ''A''	No. 47	20/65	3/10			1:8	1:256		
	79	35/120	15/45			1:2	1:32		
	114	27/75	5/17			1:16	1:128		
	Pooled	20/50	4/8			1:16	1:256		
Group ''B''	No. 73			15/30	10/25			1:32	1:64
	19			20/40	10/20			1:32	1:64
	98			12/20	5/12			1:32	1:128
	Pooled			12/40	5/9			1:16	1:128
Group ''O''	No. 5	27/75	5/15	—	5/15	1:16	1:512	1:32	1:256
	41	—	3/10	10/30	5/15	1:32	1:1024	1:16	1:1024
	46	15/60	5/20	5/15	2/3	1:16	1:256	1:16	1:128
	Pooled	15/40	3/5	10/30	3/5	1:16	1:1024	1:64	1:1024

* Before immunization.
** After immunization.
† Sub-group not determined.

specific substances (0.2 mg A + 0.2 mg B). Three weeks after the injection the individuals were bled (500 cc) and their sera (stored under aseptic conditions in the refrigerator) were examined for titer and avidity. These were compared at the same time with control samples of sera (10 cc bleedings) obtained 1 week prior to the injection of group specific substances. The titers were determined by the serum dilution technic with 1% suspensions of washed (once) red cells. The agglutination response of the serum dilutions was observed after a 15-minute period of incubation at room temperature, followed by centrifugation for 1 minute at 2000 r.p.m. Readings were made macroscopically, and the end-point reported as the greatest dilution giving a 4+ reaction, *i.e.*, complete agglutination. Avidity determinations* were obtained by mixing equal volumes of a 10% suspension of once-washed red cells and serum on a slide, which was placed on a slide-agglutination viewing box.[4] The viewing box was rocked slowly while observations were made macroscopically for the time required for the earliest visible agglutination and then for complete agglutination to develop.

The results that are summarized in Table I show the avidities and titers of the individual and the pooled lots of sera. All of the individual bleedings (numbered sera) were assayed at one time against the same red cell suspensions; the pooled sera of each group were assayed approximately one week later with other suspensions of red cells. There was a definite increase in the avidity and titer of the sera obtained from the immunized donors. The greatest increases in titer were observed in those individuals belonging to groups A and O. In the latter group sera were obtained that gave a 4+ reaction at a dilution of 1:1024 for both the anti-A and anti-B agglutinins. If the agglutination end point was taken as the greatest dilution showing some signs of agglutination, the titers of the more active sera would have values in the neighborhood of 1:4096. On the basis of a 4+ agglutination endpoint the titers obtained in our sera are at least as high as those obtained by Witebsky *et al.*[1] However, due to the variability of the quantitative agglutination test among different laboratories, such a comparison cannot be made with any certainty.[5] The greatest avidity response was obtained with the anti-B agglutinins of group A and O individuals. The pooled sera[†] in almost every case were found to have titers and avidities approximating those of the strongest sera in the group. However, this point cannot be stressed much since the pooled sera were assayed against a different batch of red cells.

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* We are indebted to Dr. L. K. Diamond of Harvard University for the technic of this determination and for the slide-agglutination viewing box that was employed.

[4] Diamond, L. K., *J. Lab. Clin. Med.*, 1945, **30**, 204.

[5] DeGowin, E. L., *J. Clin. Invest.*, 1944, **23**, 554.

Conclusions. The increase in agglutination titer following immunization confirms the work of Witebsky[1] and Wiener.[2] In addition it has been shown that immunization with group specific substances increases the avidity, and that this increase roughly parallels the increase in agglutinin titer.

[†] We are very grateful to Dr. Wm. C. Boyd of Harvard University for comparing our pooled sera with the Harvard reference standards[5] obtained as byproducts of plasma fractionation. The titers and avidities obtained by Dr. Boyd compare favorably with the reference standards. We also wish to thank Dr. Ernest Witebsky for kindly confirming the results of our assays.

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Relationship of Turbidity to Acid Production by *Lactobacillus arabinosus.*

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In microbiological assays using the *Lactobacillus arabinosus* either turbidity or total acid production may be used as a measure of the response to the organism to various growth factors. Since, in all tests for which the organism is used, the turbidity resulting from bacterial growth increases with the amount of acid produced, it has generally been assumed that the turbidity corresponding to a given amount of acid production would be the same regardless of the growth factor to which the organisms were responding. In the course of some determinations of nicotinic acid, pantothenic acid, and biotin it was noted that the turbidity was much greater in relation to acid production when the organisms were responding to suboptimal amounts of nicotinic acid than when responding to suboptimal amount of biotin or calcium pantothenate.

Methods. All tests were carried out in triplicate except those involving *l*-tryptophane[†] which were done in duplicate. The organisms were grown in Evelyn photometer tubes in 10 cc of the medium of Snell and Wright[1] as modified by Krehl, Strong, and Elvehjem,[2] omitting nicotinic acid, biotin, pantothenate, or *l*-tryptophane as the case might be. Stock cultures were carried and organisms were prepared for use as inocula as described by Snell and Wright.[1] Turbidity was measured in the Evelyn photoelectric colorimeter using filter 660. The total acid produced was titrated with 0.1 N sodium hydroxide. Lactic acid was determined by the method of Barker and Summerson.[3] These determinations were carried out in the biotin, nicotinic acid, and pantothenate tests after 18, 42, and 66 hours incubation at 37°C. In the tryptophane tests only the 66-hour determination was made and lactic acid production was not measured. The response to nicotinic acid was studied over the range from 0.1 to 1.0 μg, biotin from 0.1 to 2.0 millimicrograms, calcium pantothenate from 0.02 to 0.5 μg, and *l*-tryptophane from 2 to 10 μg per 10 ml of medium.

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[†] We are indebted to Dr. J. G. Wooley for these data.

[1] Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.

[2] Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. Eng. Chem., Analyt. Ed.*, 1943, **15**, 475.

[3] Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.

TABLE I.
Relationship of Turbidity to Acid Production by *Lactobacillus arabinosus.*

Nicotinic acid		Calcium pantothenate		Biotin		*l*-Tryptophane	
μg per 10 ml medium	Ratio* acid to turbidity	μg per 10 ml medium	Ratio* acid to turbidity	Millimicrograms per 10 ml medium	Ratio* acid to turbidity	μg per 10 ml medium	Ratio* acid to turbidity
0.1	11.95	0.02	81	0.1	51.8	2	45.8
0.2	13.0	0.04	78	0.2	51.6	4	44.4
0.4	14.2	0.1	50.8	0.6	38.3	6	42.4
0.6	16.1	0.3	24.1	0.8	36.3	8	40.0
1.0	16.9	0.5	22.0	2.0	27.2	10	39.4

* Total titratable acidity

2-Log. galvanometer reading

All figures were obtained after 66 hours incubation and are the average of triplicate determinations except in the instance of tryptophane, where they were done in duplicate.

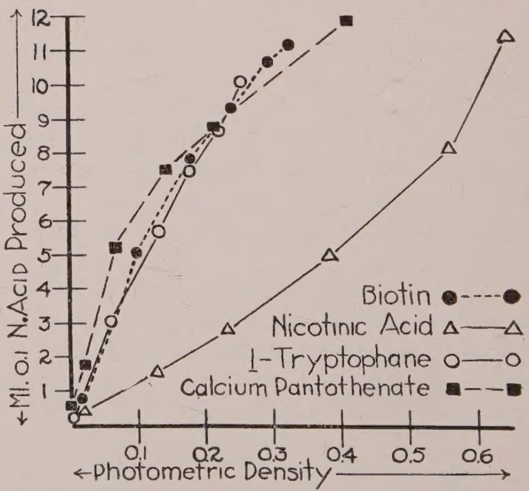
Results. The results obtained at various incubation times were similar and varied only in degree. Therefore, only the results obtained at 66 hours incubation are reported.

The ratios of total acid produced to photometric density were much lower when the organisms were responding to suboptimal amounts of nicotinic acid than when they were responding to suboptimal amounts of biotin, pantothenate, or *l*-tryptophane (Table I). At a level of acid production of 5 to 6 ml of 0.1 N acid (line 2 of Table I) the ratios were 13.0 for nicotinic acid, 78 for calcium pantothenate, 51.6 for biotin, and 44.4 for *l*-tryptophane. This indicates that, at this particular level of acid production, about 6 times as many bacteria were present in the nicotinic acid tubes as in the pantothenate tubes, and 3½ to 4 times as many as in the tryptophane or biotin tubes. As the amount of nicotinic acid was increased the ratios rose, and as the amounts of pantothenate, biotin, and tryptophane were increased, the ratios fell and tended to approach those obtained with nicotinic acid. When photometric densities were plotted against total acid production (Fig. 1), the curves for biotin, pantothenate, and *l*-tryptophane overlapped and were quite similar, whereas the nicotinic acid curve differed from all the others.

Lactic acid accounted for 98 to 102% of the total titratable acid regardless of whether the organisms were responding to nicotinic acid, biotin, or pantothenate.

Discussion. The data show that when biotin, pantothenate, or *l*-tryptophane are present in suboptimal amounts, fewer organisms are formed per unit of glucose metabolized than is the case with nicotinic acid. This suggests that lack of biotin or pantothenate interferes with the synthesis of protein to a greater degree than does lack of nicotinic acid. This view is reinforced by the similarity of the biotin and pantothenate curves to that of *l*-tryptophane. Conversely, a lack of nicotinic acid interferes with carbohydrate metabolism more than does a defi-

Figure -I-
Relationship of Photometric Density to Acid Production by Lactobacillus Arabinosus



ciency of biotin, pantothenate, or *l*-tryptophane.

Though the total fermentation of carbohydrate is reduced when biotin, pantothenate, or nicotinic acid are present in suboptimal amounts, the fact that the end product is always lactic acid suggests that the main pathways of carbohydrate metabolism are unchanged regardless of the growth factor to which the organism is responding.

Summary. 1. The turbidity produced by the growth of *Lactobacillus arabinosus* is greater in relation to acid production when the organisms are responding to suboptimal amounts of nicotinic acid than when they are responding to suboptimal amounts of biotin, pantothenic acid, or *l*-tryptophane.

2. All the acid produced by the *Lactobacillus arabinosus* is lactic acid regardless of whether the bacteria are responding to nicotinic acid, biotin, or pantothenate.

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Changes in Erythrocytes of Hamsters Following Castration, Splenectomy, and Subsequent Liver, Iron, and Testosterone Injections.

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A variety of evidence indicates that the male sex hormone has a stimulating effect upon the red blood cell count in mammals. It is well known that men have a higher average count than women, and a similar sex difference has been found to occur in many other mammals including rats,[1] rabbits,[2] mice,[3] and cats.[4] That this is due, in part at least, to the effect of the male hormone has been demonstrated by the increased count following testosterone injections in hypogonadic men,[5] in capons,[6] and in castrate female and hypophysectomized rats,[7] and by the reduction in number of red blood cells following castration in the hamster (*Cricetus auratus*) reported previously from this laboratory.[8] Some progress has also been made toward an understanding of the mechanism by which the effect of the male hormone is produced. Vollmer and Gordon[7] found increased reticulocytosis and hyperplasia of the bone marrow in hypophysectomized rats injected with testosterone propionate. The present study was undertaken to confirm and extend the results obtained following castration in the hamster[8] and also to attempt to discover further details concerning the processes involved in the effects of castration upon the red blood cells.

Experimental. Nineteen male golden hamsters (*Cricetus auratus*) (Tables I, II) were employed in this experiment, four sets of littermates plus 2 animals from the same strain but of which the exact relationship to each other was unknown. All were of the fourth and fifth generations from an original inbred litter of four animals born in this laboratory in the spring of 1943. They were kept in a temperature of 70 to 75°F and had Purina Dog Chow and/or Pratt's Nurishmix constantly available in their cages, supplemented occasionally by milk and lettuce. They appeared

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[1] Adams, A. Elizabeth, and Shevket, Fazilé, *Physiol. Zool.*, 1929, **2**, 181.

[2] Rosahn, Paul D., Pearce, Louise, and Hu, Ch'uan K'uei, *J. Exp. Med.*, 1934, **60**, 687.

[3] Kamenoff, Ralph J., PROC. SOC. EXP. BIOL. AND MED., 1936, **36**, 411.

[4] Lewis, Lena A., *Endocrinol.*, 1941, **28**, 821.

[5] McCullagh, E. P., and Jones, R., *Cleveland Clin. Quart.*, 1941, **8**, 79.

[6] Taber, Elsie, Davis, David E., and Domm, L. V., *Am. J. Physiol.*, 1943, **138**, 479.

[7] Vollmer, Erwin P., and Gordon, Albert S., *Endocrinol.*, 1941, **29**, 828.

[8] Stein, Kathryn, and Jacobsen, Barbara, *Anat. Rec.*, 1944, **88**, 459.

TABLE I.
Changes with Age in the Red Blood Cell Values of Hamsters.

	RBC count			RBC vol.			Hemoglobin		
Age in days	Animal No.	No. of animals	Avg No. RBC and range in millions	No. of animals	Total vol. in %	MCV in μ^3	No. of animals	Total Hb. g 100 cc	MCHb in $\gamma\gamma$
20	1, 2	2	5.06 (5.03-5.08)	—	—	—	—	—	—
33-34	3-7	5	5.96 (5.36-6.69)	1	47.5	70.8	—	—	—
36-37	8, 12, 13, 14	4	6.36 (5.87-6.55)	—	—	—	3	17.04	27.1
40-43	3, 7, 14	3	6.98 (6.40-7.55)	2	45.3	67.5	1	17.8	23.6
47-50	3, 7, 14	3	7.94 (7.84-8.01)	2	43.8	55.2	1	18.3	22.9
57-62	3, 7, 14-17	6	8.60 (8.16-9.29)	6	52.9	63.2	4	17.38	20.9
66-70	3, 7, 9-11	5	8.88 (8.32-9.03)	4	48.6	55.1	4	18.94	20.4
85-100	3, 7, 11, 14, 15	5	9.14 (9.06-9.02)	5	52.2	57.1	4	18.26	19.9
108-131	3, 7, 11, 14, 15	5	9.17 (9.14-9.20)	5	53.2	58.0	2	15.65	17.1

to be in healthy condition throughout the experiments.

Castration was performed on 8 animals; 1 was operated at 20 days of age, 4 at 33 to 37 days, and 3 at 66 to 73 days (Table II). Controls were subjected to similar operative procedure up to the point of actual removal of the testes. Later in the experiment 4 of the castrates, all adult and with approximately the same number of red blood cells, and 5 controls, also adult, were splenectomized (Table II). Since no significant change had occurred due to operative procedure in the controls for the castrate animals, and since the removal of the spleen was accomplished with no greater loss of blood from hemorrhage, controls for the latter series were not operated. For all the operations 0.1 cc of a 10% alcoholic solution of Nembutal (Abbott), containing 1 gram per cc, was injected intraperitoneally as a preliminary anesthetic and followed by ether as needed.

Attempts to restore the counts of operated animals to normal were made by injections of liver extract or of iron citrate. The injected animals in each of the 2 groups, liver and iron, included 2 castrates, 1 splenectomized "control," 1 splenectomized castrate, and the same normal control (Table III). The liver (from Lederle Laboratories and containing 15 U.S.P. injectable units per cc) was administered in daily intramuscular injections of 0.1 cc or 1½ units to each animal for 3 days. Counts were taken within 12 hours after the last injection. Eli Lilly iron citrate, containing 0.05 grams per cc, was injected in 0.1 cc quantities into each animal of the "iron" group daily for 3 days and counts taken within 6 hours after the last injection. Finally a series of 9 hamsters, 2 castrates, 3 splenectomized castrates, and 4 splenectomized "controls," were injected with 0.1 cc of a preparation containing one mg of propionic ester of testosterone per cc (Oreton Schering), daily for 3 days and counts taken the day following that of the last injection (Table IV).

Blood for all determinations was secured from the tails of animals under light ether anesthesia. An interval of a week was usually allowed between determinations to avoid the possibility of anemia from excess bleeding. A modified Hayem's solution recommended for rats by Smith[9] was used as the diluent. Counts were made of 4 chambers and averaged for each reported count prior to the splenectomy operations; since the coefficient of variation was usually under 1% only 2 chambers were counted thereafter. Volume determinations were made in a Van Allen capillary hematocrit centrifuged at 2600 r.p.m., and hemoglobins were read in a colorimeter equipped with a Newcomer disc.

Results. Normal Levels of Young and Adult Hamsters. The number of red blood cells in the hamster increases with age (Table I). The data confirm the results previously reported on a smaller number of animals.[8] The adult value of approximately 9 million per cc was a strikingly uniform result in all animals counted, and since it was also maintained in weekly counts up to the age of

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[9] Smith, Christianna, *J. Path. and Bact.*, 1932, **35**, 717.

TABLE II.
Effects of Castration and Splenectomy on the Erythrocyte Count.

Animal No.	Age at operation		Number of RBC in millions		
	Castration	Splenectomy	Before op.	After castration	After splenectomy
1	20 days	119 days	5.08	6.41	6.52
4	33	—	5.37	6.54	—
8	37	—	6.52	6.57	—
12	36	114	6.55	6.58	6.54
13	36	114	5.87	6.57	6.54
16	66	114	8.85	6.55	6.55
17	66	—	8.64	6.54	—
9	73	—	9.00	6.56	—
			Avg	6.55	6.54
3	—	119	9.20	—	5.73
11	—	137	9.19	—	6.52
14	—	114	9.15	—	6.54
18	—	mature	—	—	6.52
19	—	”	—	—	6.56
				Avg	6.37

165 days (Stein and Jacobsen, 226 days) it is considered as the normal level for adult hamsters of this species. Increases in total red blood cell volume also occurred with age but not proportional to the increase in number of cells; consequently the size of the individual cells was perceptibly decreased. Variations in total hemoglobin were neither great nor consistent and mean corpuscular values were higher in young animals (Table I).

Effects of Castration and Splenectomy. A level of approximately 6.5 million cells per cu mm, as compared with the normal count of 9 million, was eventually attained by all castrated animals regardless of the age at which the operation was performed (Table II). In adult animals this level was attained within a two-week period following castration and values within a range of 6.41 to 6.59 million were maintained for the 8-week period of counting. Counts for animals castrated before maturity rose to the 6.5 level and were then maintained for 1 to 6 weeks, that is, until other treatments were initiated or counting discontinued.

Removal of the spleen was followed by a marked reaction in normal control animals as compared with the lack of effect on castrates. The count for the former had dropped to an average of 6.4 million 2 weeks after the operation and this level was maintained for 6 weeks by the one animal for which weekly counts were made over so long a period. Also a

TABLE III.
Effects of Liver and Iron Injections on Red Blood Cells of Hamsters.

Animal No.	Operation	Injection	No. of RBC in millions		Size of RBC in μ^3	
			After op.	After inj.	After op.	After inj.
17	Castration	liver	6.54	9.06	—	—
8	”	”	6.55	9.14	80.9	54.7
11	Splenectomy	”	6.52	9.13	76.7	58.1
16	Castra. + splen.	”	6.54	9.19	76.3	53.3
15	Control	”	9.14	9.15	54.7	60.1
4	Castration	iron	6.54	8.85	85.6	—
9	”	”	6.56	9.11	81	—
18	Splenectomy	”	6.52	9.17	75.2	54.5
13	Castra. + splenec.	”	6.57	9.07	84.5	59.3
15	Control	”	9.14	9.21	54.7	57.5

TABLE IV.
Effects of Testosterone Injections on Red Blood Cells of Hamsters.

Animal No.	Operation	No. of RBC in millions Before testosterone	No. of RBC in millions After testosterone	Size of RBC in μ^3 Before testosterone	Size of RBC in μ^3 After testosterone
8	Castration	7.52	9.18	—	49.0
17	,,	7.73	9.12	—	—
3	Splenectomy	8.09	7.60	64.1	—
11	,,	7.63	7.62	—	61.7
14	,,	7.96	7.56	61.7	64.5
19	,,	6.54	6.52	—	65.9
1	Castr. + splen.	8.25	7.34	63.0	62.7
12	,,	7.01	7.62	65.6	64.3
16	,,	7.64	7.66	—	63.9

count of 6.49 was made on the blood of another animal dying 10 months after splenectomy.

The average size of the erythrocytes increased both after castration and after splenectomy, in the former case to 81.2 (74.5-84.1) cubic micra, in the latter to 75.4 (70.3-80.3). Normal controls had an average cell size of 56.5 (51.4-60.9) cubic micra. Splenectomy of castrates was followed by a slight decrease, to 76 cubic micra, but it is doubtful if this difference is significant. In general the effects of splenectomy were essentially the same as those of castration.

Total hemoglobin values averaged slightly higher in castrate than in control animals but the difference was too slight to be significant. The amount of hemoglobin per individual cell, however, was definitely increased, ranging from 27 to 31 micromicrograms in castrates and from 17 to 21 in controls. The effect of splenectomy upon the total amount of hemoglobin in the blood of the hamster cannot be determined at this time. Data on individual animals, "normal" and castrate, before and after splenectomy, indicate a definite drop in amount of hemoglobin per 100 cc of blood both at one and two weeks after operation. However, unoperated controls, normal and castrate, showed a similar decrease when determinations were made on the same dates as those for the splenectomized animals. This may perhaps indicate a seasonal variation in hemoglobin.

Effects of Injections. Liver or iron citrate injected into the hamsters with lower than normal red blood cell counts resulted in an immediate rise of the number to normal, whether the animals had previously been castrated, splenectomized, or both (Table III). These increases were noted within 6 to 12 hours after the last of 3 daily injections of the liver or iron. Along with the increased count a decrease occurred in the size of the individual cells (Table III).

Testosterone injected for a similar period, 3 daily injections, produced changes only in the castrated hamsters. None of the splenectomized animals, whether or not they had been castrated, showed any response in counts made the day following the last injection. The animals which received these injections had been treated previously with other substances and there had not been enough time for their counts to return to the level characteristic for their condition when the testosterone was injected. However, the effect, or lack of effect, of testosterone was obvious and consistent (Table IV).

In most of the splenectomized animals the number of red blood cells continued to drop toward the splenectomy level in spite of the testosterone injections and in none of them was there an increase in the least approaching the spectacular rise manifested by both of the castrates. The individual red blood cells remained macrocytic in all the splenectomized animals, castrated and uncastrated, injected with testosterone. In castrated "controls," however, the injections were followed by a decrease in average cell size in the one animal of this type (No. 8, Table IV) for which a volume determination was made.

Discussion. The results obtained on hamsters in this study agree with those of other workers in the field upon other animals in demonstrating a definite effect of the male sex hormone upon the number of red blood cells present in the circulating blood. As reported previously[8] castration caused approximately a 25% decrease, from 9 to 6.5 million per cu mm, in the number of cells. This is similar to the decrease from 3.6 to 2.51 million observed by Taber, Davis, and Domm[6] in capons. Injections of testosterone raised the number in castrated hamsters, as it did in capons[6] and in hypophysectomized rats.[7,10] In the latter the effect of the male hormone was noted as due to increased erythropoiesis. Because of the small amount of material available this has not been confirmed for the hamster.

Young animals responded somewhat differently from adults in that such animals, if they were castrated before the number of red blood cells had reached the adult castration level, showed an increase to that level. The final result was the same, a castration level of approximately 6.5 million cells per cu mm. The regulation of the gradual increase of the red cell count in young animals up to this level is apparently controlled by other than sex factors.

It is interesting that the change in the number of cells due to the influence of sex hormone was accompanied by a change in the size of the individual cells so that the total volume of red blood cells in the circulating blood was essentially the same in castrated as in normal animals. As far as our investigations go they seem to indicate that the total amount of hemoglobin was also kept at a relatively normal level. The increase in the amount of hemoglobin per cell in the castrates compensated on the whole for the decreased number of cells. The regulatory effect on the cell size and content thus seemed to be, in the hamster, the main effect of the male sex hormone.

The influence of the spleen on the blood picture has been the subject of numerous investigations. Both splenectomy and injections of splenic extract have indicated that the spleen may exert a stimulating effect upon the formation of red blood cells.[11,12,13] Splenectomy of hamsters in the present study produced the same decrease in the total number and the same increase in the size of individual cells as was observed following castration. This similarity and the fact that splenectomy of castrated animals caused no further changes in the red blood cells suggested a possible interrelationship between the testis and the spleen. Evidence for such a relationship was furnished by the experiments in which injections of male hormone were made. No effect of testosterone on the number or size of red blood cells was observed in splenectomized animals, whether or not they had been castrated. The effect of male hormone in changing the count was apparently not exerted directly upon the bone marrow but upon the spleen. It is possible perhaps that the increase in number following testosterone injections was due, not to an indirect effect through the spleen on erythropoiesis, but merely to a direct stimulation of the spleen to release hoarded cells. Injections over longer periods than the 3 days of the present study might give different results. However, the positive results obtained with the use of liver or iron extract after a similar short period of injection at least indicate that the bone marrow is capable of response within that period.

It is further indicated from the results of injections of liver and iron that the effect of the spleen on the number and size of the red blood cells is tied up with the liver and/or iron supply since either liver extract or iron citrate was effective, within a period of 3 to 4 days, in restoring a normal count and a normal cell size in all operated animals, castrated, splenectomized, and those which were both splenectomized and castrated. It is difficult to understand why both substances should have the same effect. Liver deficiency anemias give a macrocytic picture as compared with the small size of the cells which ordinarily occur following iron deficiency.[14] As noted previously, however, injections of iron citrate

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[10] Vollmer, Erwin P., Gordon, Albert S., and Charipper, Harry A., *Endocrinol.*, 1942, **31**, 619.

[11] Krumbhaar, E. B., and Musser, J. H., *J. Exp. Med.*, 1914, **20**, 108.

[12] Krumbhaar, E. B., and Musser, J. H., *Arch. Int. Med.*, 1923, **31**, 686.

[13] Eddy, Nathan B., *Endocrinol.*, 1921, **5**, 461.

[14] Minot, George R., *J. Am. Med. Assn.*, 1935, **105**, 1176.

as well as those of liver caused return of cell size and number to normal in the operated hamsters. It is true, of course, that the picture produced in the hamster following either or both operations may not be that of a true anemia, since the total volume of cells remains practically normal. Whether this is also true of the total hemoglobin cannot yet be stated definitely due to the complication of the results either by a seasonal variation of the pigment or by variation of technic. Krumbhaar[15] however makes no distinction in kind, but points out that there are variations in degree and in time of appearance and duration of anemia following splenectomy of different animals. He suggests that the anemia is due to a loss with the spleen of a property which aids erythrocyte formation, such as the conservation of the iron of broken down erythrocytes. And Balliere[16] noted that following splenectomy in the dog the iron content of the plasma dropped with a corresponding decrease in number of red blood cells. Presumably then the iron citrate injections may substitute for the absence of the spleen and may directly affect bone marrow. It is questionable whether the similar action of liver extract in this experiment can be attributed to its iron content since it is known that liver extract is not effective in remedying human anemia due to iron deficiency. However, perhaps the difference is a quantitative one. The only alternative explanation, if the same mechanism is supposed to be set in motion by the two types of injections, is that the injected iron has an effect on the liver of the hamster resulting in the liberation of some liver substance more directly responsible for the effects on the bone marrow. Krumbhaar[17] has also suggested that the spleen may have the mission of activating liver function.

Gordon *et al.*[18] questioned whether a function of the spleen might be to regulate the structure of the red cells as they are produced from the marrow. Certainly this study seems to indicate an influence of the spleen, and perhaps indirectly of the male hormone, on the regulation of the volume of the individual red blood cell.

Summary. Castration of hamsters caused a decrease in the number (25-30%) and in the size of erythrocytes. In animals castrated when young enough to have fewer cells than the number typical for castrates the count increased up to the castrate level. Splenectomy had the same effects as castration upon the number and size of red blood cells and no additional change occurred when castrated animals were splenectomized. Liver extract or iron citrate injected daily for three days restored a normal count and normal average corpuscular volume in all operated animals, castrates, splenectomized, and splenectomized castrates. Testosterone injected for a similar length of time was effective in castrates only and was ineffective in all splenectomized animals.

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[15] Krumbhaar, E. B., *Am. J. Med. Sci.*, 1932, **184**, 215.

[16] Balliere, A., *Biol. Abs.*, 1941, **152**, 1640.

[17] Krumbhaar, E. B., *Physiol. Rev.*, 1926, **6**, 160.

[18] Gordon, Albert S., Kleinberg, William, and Ponder, Eric, *Am. J. Physiol.*, 1937, **120**, 150.

15174 P

Sparing Action of Protein on the Pantothenic Acid Requirement of the Rat.*

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Studies on the requirement of the rat for pantothenic acid have, so far, been limited to diets containing a high proportion of carbohydrate with minimal levels of protein and fat. On these high-carbohydrate diets, it is well known that pantothenic acid deficient rats usually survive only 1 to 3 months.[1,2,3] (Table I.) The importance of the composition of the diet in relation to vitamin requirements is also well-known; *e.g.*, less thiamine is required for animals maintained on high fat[4,5] and high protein[6,7,8] diets than for diets high in carbohydrate whereas more riboflavin is required for rats receiving diets high in fat.[9,10]

In the course of experiments with diets varying in their proportions of carbohydrate, protein, and fat a marked sparing action of protein on the pantothenic acid requirement of the rat was discovered. When rats received diets deficient in pantothenic acid but containing 64% protein (casein) instead of 24% protein, growth was markedly accelerated and survival was greatly increased. This effect was observed both when the deficiency was instituted at birth and when it was instituted at weaning.

Experimental and Results. The basal (high carbohydrate) diet was composed of 64% sucrose, 24% alcohol-extracted casein, 8% hydrogenated cottonseed oil (Crisco), and 4% salts.[11] The high protein diet contained 64% casein and 24% sucrose with the remainder of the constituents identical. Both diets contained crystalline B vitamins per kg diet: 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg riboflavin, 10 mg *p*-aminobenzoic acid, 20 mg nicotinic acid, 400 mg inositol, and 1 g choline chloride. Each rat received weekly a fat-soluble vitamin mixture containing 325 mg corn oil (Mazola), 400 U.S.P. units vitamin A, 58 chick units vitamin D, and 3 mg alpha-tocopherol.

In the first series of animals, equivalent groups of male and female rats (Long-Evans strain) were placed on the deficient diets at weaning. Table I shows the marked effect of the high casein diet on growth and survival.

In the second series, litters of 6 young together with the mother were placed on the deficient diets the day of birth. The litters were weaned on the 21st day and continued on the same diet. This procedure results in a very acute deficiency[12] as can be seen in Table II. However, the sparing effect of the

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† General Mills Fellow.

[1] Mills, R. C., Shaw, J. H., Elvehjem, C. A., and Phillips, P. H., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 482.

[2] Salmon, W. D., and Engel, R. W., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 621.

[3] Unna, K., *J. Nutrition*, 1940, **20**, 565.

[4] Funk, C., *Z. f. Physiol. Chem.*, 1914, **89**, 378.

[5] Evans, H. M., and Lepkovsky, S., *J. Biol. Chem.*, 1929, **83**, 269.

[6] Banerjii, G. G., *Biochem. J.*, 1941, **35**, 1354.

[7] Banerjii, G. G., and Yudkin, J., *Biochem. J.*, 1942, **36**, 530.

[8] Wainio, W. W., *Fed. Proc.*, 1942, **1**, 87; *J. Nutrition*, 1942, **24**, 317.

[9] Evans, H. M., Lepkovsky, S., and Murphy, E., *J. Biol. Chem.*, 1934, **107**, 443.

[10] Mannering, G. J., Lipton, M. A., and Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 100.

[11] Salts No. 4 of Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

[12] Nelson, M. M., and Evans, H. M., in preparation.

TABLE I.
Effect of Dietary Protein Level on Growth and Survival in Pantothenic Acid Deficiency Instituted at Weaning.

Exp. group % protein	No. of rats	Avg wt Day 21 g	Avg wt Day 60 g	Survival Day 60 %	Avg wt Day 90 g	Survival Day 90 %	Avg wt Day 120 g	Survival Day 120 %
24	13 ♀	47	84	54	93	8	—	0
	10 ♂	48	110	80	110	50	94	10
	12 ♂	47	100	58	120	42	132	25
64	12 ♀	48	115	83	170	83	202	83
	10 ♂	47	141	90	234	90	300	90
	12 ♂	47	124	92	205	83	255	83

TABLE II.
Effect of Dietary Protein Level on Growth and Survival in Pantothenic Acid Deficiency Instituted at Birth.

Exp. group % protein	No. of rats	Avg wt Day 1 g	Avg wt Day 21 g	Survival Day 21 %	Avg wt Day 30 g	Survival Day 30 %	Avg wt Day 45 g	Survival Day 45 %	Avg wt Day 60 g	Survival Day 60 %
24	40 ♂	6.3	30	81	32	35	38	7	51	1
	38 ♀	6.1	27		31		32		—	
64	30 ♂	6.0	31	88	31	76	53	37	83	14
	30 ♀	5.9	30		30		44		73	

64% casein diet on growth and survival is still clearly shown.

The superiority of the high protein diet may actually be due to the decreased level of dietary carbohydrate inasmuch as there is evidence that pantothenic acid is involved in carbohydrate metabolism.[13] On the other hand, it may also be due to some component(s) of the casein such as (a) traces of pantothenic acid; (b) the amino acid content; (c) the phosphorus content; (d) unknown factors present which may be related to the utilization of pantothenic acid or to biotin and folic acid which affect pantothenic acid utilization.[14,15] Extensive analyses of various purified and "vitamin-free" caseins have shown the presence of pantothenic acid in all samples, varying from 0.8 to 3.0 μg per gram. However, experiments now in progress indicate that the pantothenic acid content of the casein in use is responsible for only part of the sparing action.

Summary. Rats maintained either from birth or from weaning on a pantothenic acid-deficient diet containing 64% casein grow more and survive longer than littermates maintained on a 24% casein-deficient diet under the same conditions. Several possible explanations for this sparing action are given.

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[13] Williams, R. J., *Advances in Enzymology*, 1943, **3**, 253.

[14] Wright, L. D., and Welch, A. D., *Science*, 1943, **97**, 426.

[15] Wright, L. D., and Welch, A. D., *J. Nutrition*, 1944, **27**, 55.

15175

Effect of Atabrine on *Tetrahymena geleii* (Protozoa, Ciliata).

VINCENT GROUPÉ. (Introduced by Geoffrey Rake.)

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The development during recent years of pure, bacteria-free strains of protozoa has led to more precisely controlled physiological and nutritional studies. However, the effect of chemotherapeutic drugs on such pure cultures has been little studied. It seemed of interest, therefore, to study the effect of a synthetic antimalarial drug such as atabrine on the ciliate, *Tetrahymena geleii,* whose biochemical characteristics are known.[1]

Methods. Bacteria-free cultures of *Tetrahymena geleii* were obtained through the courtesy of Professor George W. Kidder. All cultures were maintained with strict asepsis on 1.5% proteose peptone at 25°C. Strain H^1 was selected from the 6 strains tested because it was slightly less resistant to atabrine.

Population counts were made using a Howard mold counting chamber on 1.0 ml samples of culture fixed with an equal volume of formalin. Five or more fields were counted per sample using that magnification and dilution resulting in 10 to 50 cells per field. Counts were recorded as the number of intact organisms per ml.

The effect of atabrine on the division rate was determined by adding 3.0 ml of a 24-hour culture of known population diluted 1-10 in 1.5% proteose peptone to duplicate tubes containing an equal volume of a serially diluted aqueous atabrine solution. The number of generations in 24 hours was calculated from the known population at 0 hours and the average population of the duplicate tubes after 24 hours incubation at 25°C. Sterile precautions were observed throughout the entire test.

Serial dilution tests were done as follows: to 0.5 cc of serial 2-fold dilutions of atabrine an equal volume of a 24-hour culture was added. After overnight incubation at room temperature the tubes were examined both microscopically for motility and macroscopically for turbidity. Although it was most important to use bacteria-free cultures it was found possible to disregard sterile precautions in performing the serial dilution test described above without altering the results of the test.

The atabrine dihydrochloride* used in these studies was sterilized by filtration through a chemically clean UF grade sintered glass filter.

Experimental. The effect of atabrine on the division rate of *Tetrahymena geleii* was studied first to determine the concentration necessary to inhibit actively growing cells. It will be seen from the data presented in Table I that a concentration of 100 γ per ml was lethal for all cells and that multiplication was inhibited by 50 γ per ml while little or no inhibition was observed in the presence of 25 γ of atabrine per ml. In tubes containing 50 γ per ml only 25% of the population was motile. This would indicate that a difference in resistance to atabrine exists among the population. Similar results were obtained in another experiment.

Inasmuch as direct observation showed that loss of motility was followed rapidly by swelling and disintegration of the cell in the above experiments, a simple serial dilution test (see methods) was used to determine the effect of age of the culture on the resistance of the cells to atabrine. Population counts, pH, and relative resistance to atabrine were determined on samples taken daily from each of 3 flask cultures over a period of 4 days. Comparable results were obtained on all 3 cultures and the pertinent data from one such culture is presented in Table II. It will be seen that the concentration of atabrine required to immo-

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[1] Kidder, G. W., and Dewey, V. C., *Physiol. Zoöl.*, 1945, **18**, 136.

* The atabrine dihydrochloride used was obtained through the courtesy of Dr. Arthur P. Richardson, Head of the Division of Pharmacology of the Squibb Institute for Medical Research.

TABLE I.
Effect of Atabrine on Division Rate.

Atabrine, gamma per ml	Intact organisms per ml		Calculated No. of generations in 24 hr	% of population motile after 24 hr
	0 hr	24 hr		
400	4300	0	—	0
200	''	0	—	0
100	''	0*	—	0
50	''	3,000	—0.5	25
25	''	24,000	2.5	100
12.5	''	24,400	2.5	100
6.3	''	24,000	2.5	100
3.1	''	31,200	2.8	100
1.6	''	33,200	3.0	100
0.8	''	39,000	3.2	100
0	''	37,000	3.1	100

* No growth on subculture.

TABLE II.
Effect of Age of Culture on Resistance to Atabrine

Age of culture, Hr	Population, cells/cc × 1000	pH	Atabrine—γ per ml								
			250	125	62.5	31.3	15.6	7.8	3.9	1.95	0
1	2	6.9	0	0	1+	4+	4+				4+
24	30.6	7.1	0	0	0	1+	4+				4+
48	43.4	7.2	0	0	0	0	0	4+	4+		4+
72	127	7.6	0	0	0	0	0	±	4+	4+	4+
96	114	7.7	0	0	0	0	0	±	4+	4+	4+

0 = No motile cells observed.
± = Occasional motile cell.
1+ = Approximately 25% of cells motile.
2+ = '' 50% '' '' ''
3+ = '' 75% '' '' ''
4+ = All cells motile.

bilize all cells progressively decreased from 125 γ per ml 1 hour after the flask was inoculated to 15.6 γ per ml, 72 hours after inoculation. In order to determine what factors might be responsible for this marked decrease in resistance to atabrine the experiments described below were carried out.

Size of the population did not appreciably affect resistance to atabrine since a 32-fold dilution of washed cells from a 24-hour culture had little or no effect on the concentration of atabrine required to immobilize the cells. However, as shown in Table II, the pH increased from 6.9 to 7.6 during 72 hours of incubation. Since an increase in pH resulted in more effective inhibition of *Escherichia coli* by atabrine,[2] it was of interest to study the effect of the pH of the culture on the resistance of *Tetrahymena geleii* to atabrine. It will be seen (Table III) that the resistance of a 24-hour culture to atabrine decreased at least 1,000-fold when the pH of the culture was increased from pH 6.35, where the cells survived a concentration of 5,000 γ or more of atabrine per ml, to pH 7.48 where only 4.9 γ per ml were required to immobilize all cells. In each of 2 additional experiments resistance to atabrine was strikingly decreased when the pH was increased although it was not possible to predict the lowest inhibitory concentration of atabrine from the pH of the culture. It was also found that when a 9-day-old culture at pH 7.8 was adjusted to pH 6.9 the resistance to atabrine only increased from 4 to 16 γ per ml.

It was of interest to determine whether, aside from the increase in pH, the medium was altered during growth by the accumulation of metabolic products or cellular debris in such a way as to decrease the resistance of the cells. The results of a single experiment pre-

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[2] Silverman, M., and Evans, E. A., *J. Biol. Chem.*, 1944, **154**, 521.

TABLE III.
Effect of pH of Culture on Resistance to Atabrine.

pH of culture	Atabrine—γ per ml													
	5,000	2,500	1,250	625	313	156	78	39	19.5	9.8	4.9	2.5	1.3	0
6.35	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
6.60	1+	1+	1+	2+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
6.80	0	0	0	0	0	0	4+	4+	4+	4+	4+	4+	4+	4+
6.95	0	0	0	0	0	0	0	±	4+	4+	4+	4+	4+	4+
7.18	0	0	0	0	0	0	0	0	0	0	4+	4+	4+	4+
7.48	0	0	0	0	0	0	0	0	0	0	0	3+	4+	4+

TABLE IV.
Effect of Conditioned Medium and Frozen Cells on Resistance to Atabrine.

		Atabrine—γ per ml							
24-hr culture plus an equal volume of:	pH	500	250	125	63	31	16	8	0
Fresh medium	7.0	0	0	0	1+	4+	4+	4+	4+
Conditioned medium*	7.0	0	0	0	0	0	4+	4+	4+
Frozen cell suspension† (cellular debris)	7.0	0	0	0	0	0	2+	4+	4+

* Cell-free supernate from 6-day-old culture adjusted to pH 7.0.
† 24-hour-old cells resuspended in ⅓ volume of fresh medium then frozen and thawed.

sented in Table IV show that at pH 7.0 the resistance of a 24-hour-old culture was significantly decreased by the addition of an equal volume of conditioned medium (obtained by adjusting the cell-free supernate from a 6-day-old culture to pH 7.0) or a frozen cell suspension containing only cellular debris.

That the decreased resistance of cells from older cultures is the result of environmental changes is shown by the fact that when 24-hour- and 9-day-old cells were resuspended in fresh medium to contain 122,000 and 140,000 cells per ml respectively at pH 6.9 a concentration of 63 γ of atabrine per ml immobilized approximately 50% of the population in each case.

Although, as shown in the experiments described above, the resistance of *Tetrahymena geleii* to atabrine was influenced by many factors it was possible to obtain quite reproducible results using 24-hour bacteria-free cultures in the logarithmic phase of growth at pH 6.9 to 7.0. A test similar to that used for the quantitative determination of antibiotic activity[3] was used and performed as follows. Two ml of culture was added to a series of Wassermann tubes each of which contained 20% less atabrine than the preceding tube. After overnight incubation at 25°C the endpoint was recorded as the highest dilution of atabrine in which no macroscpic turbidity was observed (although motile cells were present). Providing a bacteria-free culture was used the endpoint was not affected when sterile precautions were disregarded in performing the test. In a series of 14 replicate titrations performed over a period of one month, according to the technic described above, an endpoint of 40 to 43 γ of atabrine was obtained in every case. Bacteria-free cultures of *Tetrahymena geleii* are particularly well suited for such studies because conjugation does not occur and endomixis has not been observed.[4]

Summary. Multiplication of *Tetrahymena geleii* was inhibited by 50 γ of atabrine per ml. Resistance of the cells to atabrine was progressively decreased by the environmental changes occurring during growth of the culture and was strikingly decreased when the pH of the culture was increased.

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[3] McKee, C. M., Rake, G., and Menzel, A. E. O., *J. Immunology*, 1944, **48**, 259.

[4] Kidder, G. W., personal communication.

15176

Therapeutic Effect of Streptomycin in Experimental Murine Pertussis.*

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Streptomycin, an antibiotic discovered by Waksman and his associates, is produced by the growth of certain strains of *Actinomyces griseus* in a suitable medium.[1] Because it exhibits activity against certain gram-negative organisms and is relatively non-toxic, it seemed to us important to test its value in the treatment of experimental murine pertussis as a preliminary to its use in the human disease. The present report describes the therapeutic effect of streptomycin on the course of the experimental disease in mice.

Materials and Method. A standard type of experimental infection in mice,[2] used for several years in this laboratory, was employed. It consisted of the intranasal inoculation of groups of standard 3-week-old white Swiss mice with freshly isolated strains of *Hemophilus pertussis*.

The 48-hour growth of *H. pertussis* was scraped from the surface of Bordet medium into 0.85% salt solution, thoroughly mixed, strained through gauze and standardized to contain approximately 10 billion organisms per ml. Four-fold dilutions of this suspension were prepared for the inoculation of subgroups of mice. Under light ether anesthesia, each mouse, held in the upright position, received an intranasal instillation of 0.05 ml of the organism suspension. Usually 5 subgroups, each consisting of 4 mice, were inoculated. The dose of organisms given to the mice of the various sub-groups was approximately 125, 31.2, 7.8, 1.9, and 0.47 million respectively.

After inoculation the mice were kept under uniform environmental conditions. Deaths were recorded daily for a 10-day period, at the end of which period the surviving mice were autopsied and cultures of the lungs made. The method of Reed and Muench[3] was used to calculate the 50% endpoint, indicating the minimal lethal dose (MLD) for each group of 20 mice.

Streptomycin hydrochloride (Lot 228) was supplied for these experiments by Merck and Co. through the courtesy of Dr. D. F. Robertson at the request of Dr. S. A. Waksman who had kindly supplied us with material for preliminary experiments. It contained 350 units per milligram of dry material and was dissolved and diluted to appropriate concentration in sterile distilled water. Treatment was instituted 4 hours after the mice were infected.

Sodium penicillin (Lot 299) (Cheplin), in the crystalline form, contained 100,000 Oxford units per 60 mg and was prepared for injection in a similar manner.

Results. Under the conditions of the experiments it is clear that streptomycin was definitely protective against the experimental disease. As indicated by the data (Table I), it required more than 10 times the number of organisms (Schieven Strain) to kill 50% of the treated mice than the untreated. When another strain (Rizzo) was used, the protection ratio was more than 3 to 1 in favor of the treated group. In addition to the difference of the mortality rates between the treated and untreated groups there was a distinct difference between the percentages of surviving mice showing negative lung cultures.

In the first experiment (Table I) 14 (73%) of the 19 surviving treated mice had negative lung cultures, indicating a rapid clearance of the organisms from the lungs as a result of treatment with the antibiotic. Only 2 (18%)

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* Supported in part by a grant from The John and Mary R. Markle Foundation.

[1] Schatz, A., Bugie, E., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 66.

[2] Bradford, W. L., Brooks, A. M., and Katsampes, C. P., *Yale J. Biol. and Med.*, 1944, **16**, 435.

[3] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
Therapeutic Effect of Streptomycin in Experimental Murine Pertussis.

	No. of survivors in each subgroup of 4 mice Dose of organisms in millions					M.L.D. of millions of organisms	% of survivors with negative lung cultures	Ratio of difference in % to its S.E.
Exp. I (Schieven Strain)	125	31.2	7.8	1.9	0.47			
Streptomycin*	3	4	4	4	4	>125	73	3.4 to 1
Saline (controls)	0	1	4	3	3	12.7	18	
Exp. II (Rizzo Strain)								
Streptomycin*	4	4	4	4	4	>125	100	2 to 1
Saline (controls)	0	3	3	4	4	39	78	

* Lot 228—0.2 ml (130 units) injected subcutaneously 4 × daily for 4½ days. Total dose for each mouse: 2340 units.

† Lot 228—0.25 ml (250 units) injected subcutaneously 4 × daily for 5 days. Total dose for each mouse: 5000 units.

TABLE II.
Therapeutic Effect of Streptomycin and of Penicillin in Experimental Murine Pertussis.

	No. of survivors in each subgroup of 4 mice Dose of organisms in millions					M.L.D. of millions of organisms	% of survivors with negative lung cultures	Ratio of difference in % to its S.E.
(Sharpe Strain)	125	31.2	7.8	1.9	0.47			
*Streptomycin	4	4	4	4	4	>125	100	4.4-1
†Penicillin	0	0	1	4	2	3.3	29	—
‡Streptomycin and penicillin	4	4	4	4	4	>125	95	4.0-1
Saline (controls)	0	0	1	1	3	1.2	20	

* Lot 228—Each mouse received intraäbdominally 0.25 ml (250 units) 4 × daily for 5 days. Total dose: 5,000 units per mouse.

† Lot 299—(Cheplin) Each mouse received intraäbdominally 0.25 ml (5 units) 4 × daily for 5 days. Total dose: 100 units per mouse.

‡ Each mouse received intraäbdominally 5 units of penicillin and 250 units of streptomycin 4 × daily, for 5 days. Total dose: 100 units of penicillin + 5,000 units of streptomycin.

of 11 untreated survivors had negative cultures. This difference in the percentages of negative cultures obtained from the 2 groups is statistically significant, for the ratio between the difference and its standard error is 3.4 to 1. In the second experiment (Table I) the clearance effect was similar, though not so definite as in the first experiment (ratio: 2 to 1).

The data of Table II concerns a third experiment in which the protective effect of streptomycin was compared with that of penicillin. It is clear that streptomycin was again definitely protective while penicillin failed. When the 2 antibiotics were combined, the result was similar to that of streptomycin alone.

The protective action of streptomycin as indicated in the above experiments was sufficient to justify a trial in the human disease, studies of which are now in progress.

Summary. Streptomycin exerts a protective therapeutic effect on the course of experimental murine pertussis as indicated by a significant reduction in the mortality rate and the disappearance of the organisms from the lungs of the surviving mice.

15177 P

Electric Control of Polar Growth in Roots of *Allium cepa.*

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Numerous experiments have been performed to determine the effects of continuous electric currents passed through growing roots immersed in various aqueous media. In practically all, the electric field was oriented at right angles to the long axis of the root. Under such conditions a minimum of two diverse stimuli, *viz.*, gravity and applied E.M.F., operate at right angles to one another, thus causing a complicated type of variable growth response.[1,2]

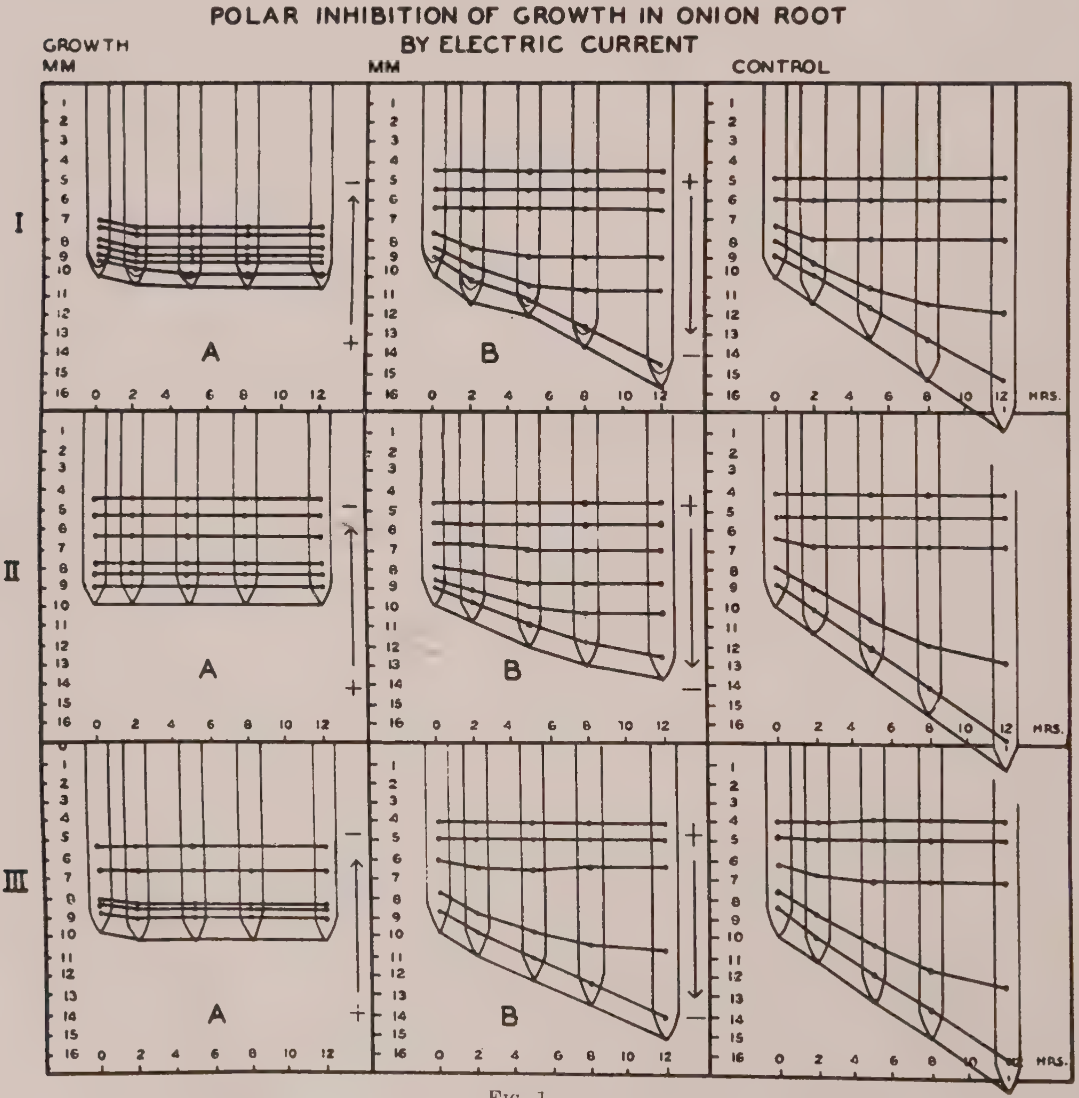

Fig. 1.

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[1] Elfving, F., *Bot. Z.*, 1882, **40**, 258.

[2] Navez, A. E., *J. Gen. Physiol.*, 1927, **10**, 551.

To provide simpler experimental conditions, an electric current having parallel isopotential lines and appropriate current density must be passed up or down the polar axis of the root and must coincide with the orientation of the general axis of the inherent electric field of the root as well as that of gravity.

An apparatus was designed to comply with this requirement and to maintain the roots under normal conditions of growth except for the applied current. Three sets of roots were used in each experiment: (1) control roots, (2) roots through which an electric current was passed upward, and (3) roots in which the same current was passed downward. Since individual roots vary slightly with respect to the threshold of current density for reversible inhibition of growth and also in their rates of elongation, each experiment involved 3 or more duplicate sets of roots. Preliminary experiments led to a choice of a current giving an IR drop of 40 mV/mm, with the root immersed in tap water as the growing medium and the current passed upward through 25 mm of the apical end of the growing root. This gives complete inhibition of growth. The same current passed downward gives a very slight inhibition of growth, Fig. 1, or none at all. At 37.8 mV/mm, complete but reversible growth inhibition is obtained with ascending currents.

Most experiments were divided into 3 periods: normal growth rate, current passing, and current off. The growth of each root was recorded under the microscope throughout the experiment.

Regions of the Growing Root Affected by the Electric Current. To determine what region of the growing root apex is involved in the growth response and inhibited by the current, a set of roots was marked in the usual way by means of particles of India ink. The results of one experiment are drawn to scale in Fig. 1. Ordinates are mm length, abscissa hours. I, II, and III are triplicate sets. A current of 40 mV/mm was passed up roots A, down roots B, and the right-hand group were controls. It is evident from the measurements that the growth process under these conditions was prevented by an ascending current in both the region of active cell division and the region of cell elongation.

The inherent E.M.F.'s generated by the root are so oriented that the ascending current, which causes inhibition, opposes them. An applied current (field) of like intensity, but oriented with, rather than against the inherent one, has little or no effect. A detailed report of all experiments will be presented elsewhere.

15178 P

Effect of Induced Pain on Pain Threshold.*

C. M. PARSONS AND F. R. GOETZL. (Introduced by Harold S. Olcott.)

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Relief of pain by counterirritation is common experience and frequently was utilized therapeutically by the older physicians. The number of experimental studies reported in the literature is too small to permit conclusions to be drawn regarding mechanisms involved in the phenomenon which thus remains but poorly understood. Therefore reinvestigations on a larger group of subjects appeared desirable.

Methods. Pain threshold determinations were performed in a group of 14 normal human subjects, men and women, ranging in age between 19 and 45 years. The algesimetric method employed has been described in detail elsewhere.[1] It consisted of applying an

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[*] This work was aided in part by a grant from Whitehall Pharmacal Company.

[1] Goetzl, F. R., Burrill, D. Y., and Ivy, A. C., *Quart. Bull. Northwestern Univ. Med. School*, 1943, **17**, 280.

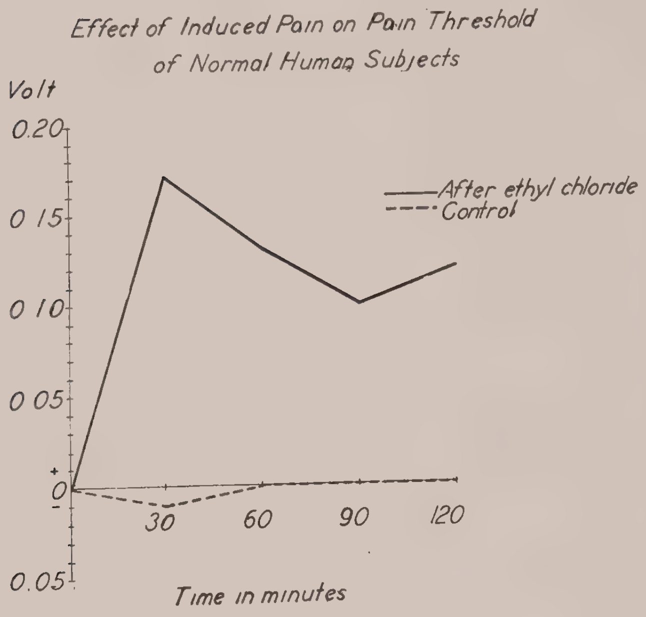

Fig. 1

alternating current to a tooth through a metal filling and noting the voltage at which the subject first experienced a painful sensation. Such voltage was considered an expression of the pain threshold level.

Pain was produced by spraying ethylchloride to an area of one square inch over the anterior surface of the subjects' tibiæ for a period of 20 seconds. At that time pain of burning or aching character was complained of in all instances. Pain thresholds were determined shortly before and at 30-minute intervals for 2 hours after the application of ethylchloride.

Results. Fig. 1 shows the averages of the values obtained in the determinations of pain threshold, expressed in terms of the mean changes in voltage required to produce a painful sensation in the subjects' teeth. The control values were obtained from 34 control tests in the 14 subjects and were found to be between 0.2 and 1.8 volt. Values after application of ethylchloride were obtained from 21 experiments in 11 of the 14 subjects. Analgesic effects were observed in all instances. The greatest variation in pain threshold during control periods in every subject was significantly smaller than the smallest change of threshold following application of ethylchloride. Therefore the curves presented give an indication of the results obtained for each subject.

In the control determinations the mean change in threshold value for the entire group was minus 0.003 volt; the corresponding value for the period following application of ethylchloride was plus 0.129 volt.

Comment. The observations reported are in partial agreement with results obtained by other authors.[2] They, however, employed different algesimetric procedures and different types of induced pain. Also, their studies were made on only 3 subjects and the results were expressed in percentages rather than in actual changes in threshold values. The pain threshold was reported to be elevated by induced pain by approximately 30%.

It is interesting, also, to compare the present results with those obtained previously in studies on analgesic effects of morphine sulfate in normal human subjects using the present method.[3] Not all of the subjects tested showed an elevation in pain threshold following administration of 0.016 g of morphine sulfate, which for the entire group produced a mean increase of only 0.0218 volt.

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[2] Hardy, J. D., Wolff, H. G., and Goodell, H., *J. Clin. Invest.*, 1940, **19**, 649.

The fact that for producing analgesic effects in the present experiment ethylchloride had to be applied until pain developed indicated that pain rather than cold was the sensation responsible for elevating pain thresholds. The mechanisms involved in producing the analgesic effects cannot yet be stated with certainty. Distraction, changes in local blood supply and other short lasting effects of induced pain cannot fully explain the phenomenon because the pain induced in our subjects lasted for 2 to 3 minutes at the most while the analgesic effects were noticeable for a period of 90 to 120 minutes.

In additional investigations, the possibility is being examined whether epinephrine might be the agent involved in producing analgesic effects following induced pain. The trend of thought seems logical in view of the facts that epinephrine is released by pain from the adrenal glands[4] and that it possesses marked analgesic properties when injected in man or dog.[5]

Summary. Experiments have been made in normal human subjects which demonstrate that induced pain of short duration produces long lasting analgesic effects.

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[3] Ivy, A. C., Goetzl, F. R., and Burrill, D. Y., *War Medicine*, 1944, **6**, 67.

[4] Cannon, W. B., *Bodily Changes in Pain, Hunger, Fear, and Rage*, New York, D. Appleton and Co., 1929.

[5] Ivy, A. C., Goetzl, F. R., Harris, S. C., and Burrill, D. Y., *Quart. Bull. Northwestern Univ. Med. School*, 1944, **18**, 298.

15179

Effect of Adrenocorticotropic Hormone (ACTH) on Hypophysectomized Adrenal-demedullated Rats.*

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From the Institute of Experimental Biology, University of California, Berkeley.

Ingle[1] has raised the question of the possible role of the adrenal medulla in the response of the adrenal cortex to adrenocorticotropic hormone. In the present study the problem of the relationship between the two parts of the adrenal has been approached by comparing the effectiveness of adrenocorticotropic hormone as a stimulant of the cortical tissue when administered in the presence or absence of the medullary tissue.

Methods. Male rats were adrenal-demedullated† at 25 days of age and the adrenal cortex was allowed to regenerate for 16 days. The animals were supported for the first 13 days of this period by 1% NaCl in the drinking water. The rats were then hypophysectomized, at 41 days of age. Pure ACTH[2] was injected for 15 days, beginning on the day of hypophysectomy, in an effort to maintain the adrenal cortical tissue. The cortical response of the doubly operated animals was compared with that of an equal number of rats hypophysectomized at the same age, in which the adrenal was intact. The daily dose of ACTH, 0.2 mg, was divided into two intraperitoneal injections of 0.5 cc each.

At autopsy the sella turcica of all hypophysectomized rats was searched under 10-fold magnification for remnants of pituitary tissue.

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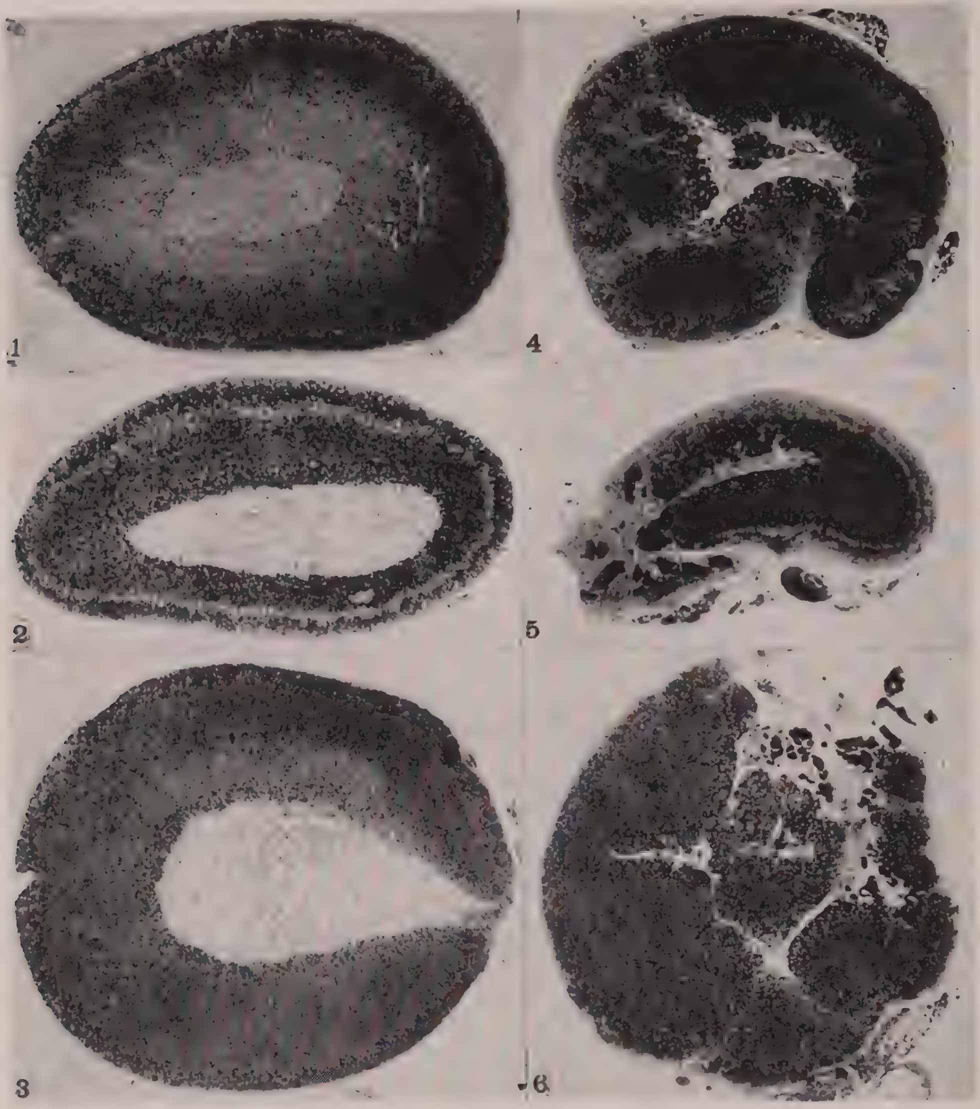
* Aided by grants from the Research Board of the University of California, the Rockefeller Foundation, and the Josiah Macy, Jr., Foundation.

[1] Ingle, D. J., *Endocrinol.*, 1942, **31**, 419.

† The adrenal medulla and all cortical tissue except a few cells adhering to the capsule were enucleated.

[2] Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1943, **149**, 413.



Sudan orange stain, X 21.5.

FIG. 1. Adrenal of a normal 41-day-old male rat, illustrating the condition of the adrenal cortex at the time of hypophysectomy. Note the even distribution of fine lipid droplets, with a slightly greater concentration in the glomerulosa and a faint subglomerular zone of somewhat depleted lipid content. Weight of 2 adrenals 25 mg.

FIG. 2. Adrenal of a 56-day-old hypophysectomized male rat, 15 days postoperative. The cortex is markedly reduced in width. The lipid occurs in coarse droplets and there is a wide subglomerular sudanophobe zone. Weight of 2 adrenals 10 mg.

FIG. 3. Adrenal of a 56-day-old hypophysectomized rat treated with 0.2 mg of pure ACTH daily for 15 days, beginning on the day of hypophysectomy at 41 days of age. The cortex has been completely maintained and even slightly hypertrophied, as shown by the strikingly uniform distribution of lipid throughout its entire width, obliterating even the narrow subglomerular zone of the normal male. Weight of 2 adrenals 22 mg.

FIG. 4. Regenerated adrenal cortex of a 41-day-old male rat which was adrenal-demedullated at 25 days of age. The cortical tissue has reorganized in the normal zonal relationships around a central vein. The narrow subglomerular zone of lipid depletion characteristic of the normal male is discernible. Weight of cortical tissue 12 mg.

FIG. 5. Regenerated adrenal cortex of a 56-day-old male rat which was adrenal-demedullated

at 25 days of age and hypophysectomized at 41 days of age. During the 15 days elapsing since the second operation, the typical pattern of hypophysectomy has been superimposed upon the regenerated cortex. Weight of cortical tissue 8 mg.

FIG. 6. Regenerated adrenal cortex of a 56-day-old male rat which was adrenal-demedullated at 25 days of age, hypophysectomized at 41 days of age and treated, beginning immediately after operation, with 0.2 mg of pure ACTH daily for 15 days. The cortical lipid distribution is entirely comparable to that seen in the injected hypophysectomized animal with intact adrenals, and the same evidence of hypertrophy is seen in the obliteration of the subglomerular zone (Fig. 3). Reorganization of the regenerated cortical tissue has advanced as much under the influence of ACTH as in the presence of the pituitary. Weight of cortical tissue 17 mg.

Any questionable tissue was serially sectioned and examined. Atrophy of the thyroid and gonads gave additional evidence for complete removal of the pituitary. The adrenal tissue of the "demedullated" rats was sectioned serially and examined for remnants of medullary tissue. Any animals in which fragments of pituitary or adrenal medullary tissue had been left at operation have been eliminated in reporting the results of the experiment.

A total of 42 rats was used. It was necessary to provide appropriate controls for each important period in the experiment, in order to establish: (1) the extent of cortical regeneration 16 days after adrenal demedullation, at which time hypophysectomy was performed and injections were begun; (2) the condition of the adrenal cortex of the normal animal at that time; (3) the effect of hypophysectomy upon both regenerated and normal cortical tissue, as seen 15 days postoperatively, at the end of the experiment; (4) the amount of additional cortical regeneration which had taken place in the demedullated animal possessing a pituitary, during this 15-day period. Accumulated data on normal 56-day-old rats were available for the controls at the end of the experiment.

The distribution of animals into experimental and control groups may be seen in Table I. Initially there were 6 animals in each group.

Results. As will be seen in Table I, there was no difference in cortical weight response to ACTH conditioned by the presence or absence of medullary tissue. The dose used, 0.2 mg daily, was adequate to maintain the adrenal weight at the time of hypophysectomy in both types of experimental animal. The thymus was also weighed as a further measure of cortical response, and its reduction was found to have been equally drastic in both types of animal receiving ACTH.

Frozen sections of the adrenals, stained with Sudan orange, are illustrated in Fig. 1 to 6, and the salient features of cortical lipid distribution are described there. The regenerated cortical tissue in demedullated adrenals was protected against the effects of hypophysectomy by this dose of ACTH as completely as was the cortex of intact adrenals (Fig. 3 and 6). Although the weights had not been increased above the operative level, the adrenal cortex in both hypophysectomized and doubly operated animals showed some evidences of hypertrophy. Vividly sudanophilic lipid, in droplets slightly larger than normal, was distributed evenly throughout the entire width of the cortex, obliterating even the narrow subglomerular zone characteristic

TABLE I.
Response of Hypophysectomized Rats to ACTH in the Presence or Absence of the Adrenal Medulla.

	Adrenals intact				Adrenals demedullated			
Treatment	No. of rats	Body wt, g	Adrenals wt, mg	Thymus wt, mg	No. of rats	Body wt, g	Cortical tissue, mg	Thymus wt, mg
Controls at time of hypophysectomy, 41 days old	6	133	25	364	6	139	17	402
Hypophysectomized, ACTH for 15 days	6	105	24	63	6	115	17	91
Hypophysectomized controls at autopsy, 15 days postoperative	6	115	10	196	6	124	10	199
Controls at autopsy, 56 days old	54	215	34	451	5	215	22	436

of the normal male.[‡] The cortical tissue of the ACTH-treated doubly operated animals seemed, in addition, to have undergone reorganization beyond that seen at the time of hypophysectomy; it resembled more closely the condition in the demedullated controls at autopsy, in which the cortical tissue had been continuing regeneration under the influence of the animal's own pituitary.

Conclusions. The stimulating action of ACTH on the adrenal cortex of hypophysectomized rats, as far as could be judged by adrenal weights and by morphology as seen in lipid stains, was the same in the absence of the medulla as in the intact adrenal.

[‡] This is the typical picture of adrenal hypertrophy which has been obtained upon administration of adequate doses of ACTH to normal, hypophysectomized, gonadectomized, hypophysectomized-gonadectomized or hypophysectomized-thyroidectomized male rats.

15180 P

Hypertension Produced in Dogs by Unilateral Ligation of Periadrenal Blood Vessels and Tissue.

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The possibility that adrenal ischemia may cause hypertension was investigated in the present experiments which are concerned with the effect of decreased adrenal blood supply upon systemic blood pressure.

Method. Mongrel dogs were employed. Systolic blood pressure was determined indirectly by a mercury manometer attached to an inflated cuff wrapped around the hind leg just above the ankle of trained unanesthetized dogs. Systolic pressure was indicated by the pressure at which pulsation of the dorsalis pedis artery was first palpable when the air in the cuff was released. The cuff was inflated 15-30 successive times to determine the range of systolic pressure. These observations were checked by direct measurements with the Hamilton manometer with readings from the femoral artery and showed agreement within 5-15 mm Hg. Animals were housed in separate cages. Blood pressures were recorded daily for a preliminary period of 3-6 weeks. Then either a sham operation in the adrenal area or one to decrease adrenal blood flow was performed on one adrenal. Thereafter blood pressures were taken daily. Urine analysis and blood NPN levels were obtained before and after operation.

Operation. The left adrenal was chosen because it was more easily exposed than the right. Blood vessels come to the left adrenal from the aorta to the hilum, from the diaphragm to the superior pole and from below to the inferior pole. Many arteries enter the adrenal from pericapsular fat and connective tissue. Because of the extreme vascularity of the tissue around the adrenal it seemed that an operation which would constrict or obstruct large numbers of vessels around the capsule would be most favorable for diminishing blood supply. Two ligatures of No. 13 white surgical silk braid were tied about vascular fat and connective tissue adjacent to the adrenal. One was placed around the hilar artery and vein and the other around grossly visible arteries and veins at either the superior or inferior pole. The area involved in the ligature was about 1.0 x 0.5 cm. The procedure just described was used on 5 dogs. In a sixth, No. 391, an artery to the inferior pole and the hilar vein were stripped of fat and connective tissue and ligated with No. 11 surgical silk. To rule out non-specific effects of these procedures, sham operations were performed in 3 dogs, 2 of which had subsequent operations for producing adrenal ischemia. A ligature of

TABLE I.
Hypertension Produced in Dogs by Unilateral Ligation of Periadrenal Tissue and Blood Vessels.

Dog No.	Systolic blood pressure, mm.Hg.					
	Before ligation	After sham operation	After ligation	Months after ligation	Blood pressure, mm.Hg. Systolic/diastolic	NPN
361	100-150		180-261	6	261/119	34
384	125-190		190-262	7	248/102	32
385	130-190		195-267	7	267/112	30
386	110-200	130-210	190-253	4	253/130	29
387	120-140	110-150				
390	110-170	130-170	210-280	4	241/131	36
391	130-190		176-255	2	255/108	33

the same material described above was placed through the peritoneum over the surface of the adrenal. Care was taken not to include any grossly visible blood vessels.

Results. Dogs, 4 female and 1 male, subjected to unilateral ligation of periadrenal blood vessels and tissue developed within 1-3 days elevation of blood pressure which has persisted to the present, 2-7 months later. Another male dog, No. 391, had hypertension after ligation of one artery running to the lower pole of the gland and of the hilar vein. Both systolic and diastolic pressure were elevated in all cases, the latter being over 100 mm Hg. and the former going as high as 280 mm. Hg. (Table I).

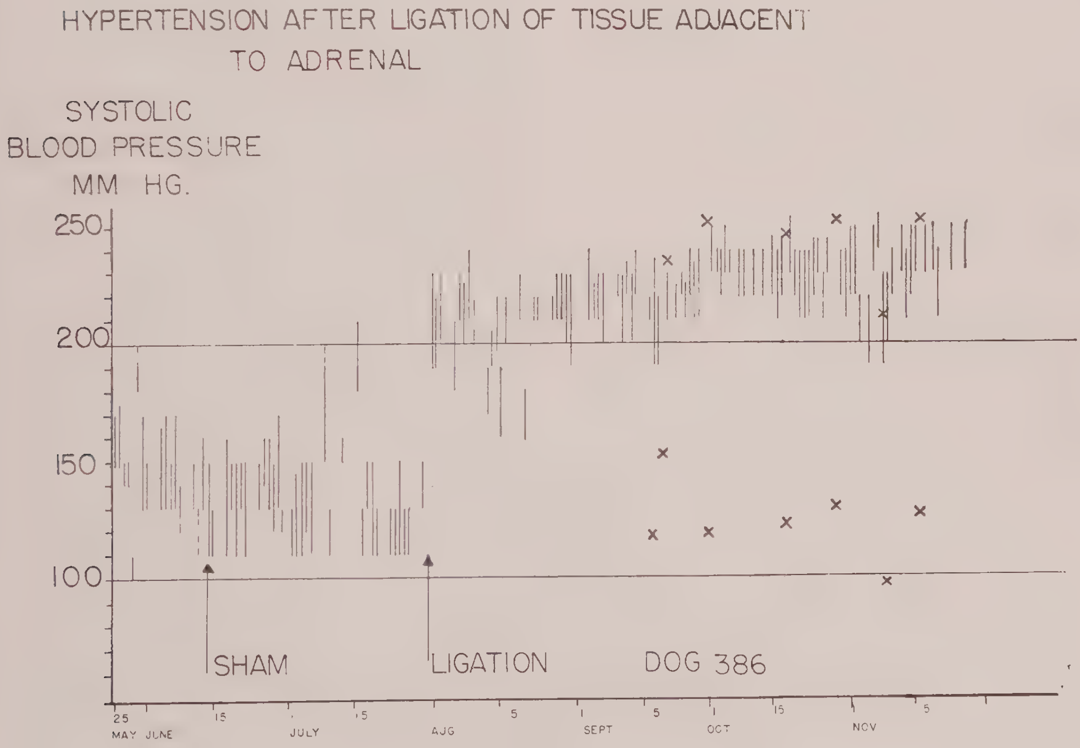

FIG. 1.

Vertical lines indicate ranges of systolic blood pressures. Upper and lower crosses (×) represent systolic and diastolic pressures respectively. Sham operation on right adrenal had no effect but ligation of left periadrenal tissues and blood vessels produced hypertension.

Sham operation produced no change in systolic blood pressure in 3 dogs which were followed for 6 weeks. Subsequent operations in which periadrenal blood vessels and tissues were ligated in dog No. 390 and No. 386, Fig. 1, caused elevation of blood pressure which has persisted until the present, 4 months later. To date, there have been no significant changes in the urine analysis or blood NPN levels of the hypertensive dogs.

Conclusion. A prompt and sustained hypertension was produced in 6 dogs by unilateral subtotal ligation of periadrenal blood vessels and tissue.

It is a pleasure to thank Miss Goldie Spierer and Mr. Dominick Triolo for their assistance in these observations.

15181

The Production of Shock in Rats by the Drum Method.*

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Noble and Collip[1] described a method by which rats were subjected to graded degrees of trauma by tumbling in drums for varying periods of time. Animals subjected to this type of trauma did not develop complications of hemorrhage and infection. The undesirable factor of an anesthetic was likewise eliminated. Hemoconcentration and other pathological changes ascribed to the condition of shock did develop. These workers found that the drum method produced a better graded and controlled type of trauma than any other method they tried, and made use of it in subsequent experiments.[2,3] Zahl, Hutner, and Cooper[4] used the method and confirmed Noble and Collip's results as to the number of turns, percentage of deaths, survival time, and reproducibility. Chambers, Zweifach, and Lowenstein[5] employed the method to study circulatory reactions of rats, and Clarke and Cleghorn[6] used the drug method to study tissue potassium and phosphorus in shocked rats. In our laboratory we were interested in finding a method for producing shock which would give as nearly uniform and reproducible results as possible in animals to be used for biochemical studies. We wished to determine the chemical changes in the blood and tissues of animals in shock, and in animals which had received sublethal stimuli under conditions which could be readily controlled so that a quantitative relationship between the sublethal and lethal stimuli could be defined. As the tissues were to be analyzed for extremely labile substances they had to be frozen *in situ* with liquid air. The necessity for rapid freezing precluded the use of any animal larger than a rat. The method of Noble and Collip for production of shock was chosen because it seemed best to meet the requirements for the chemical investigations and also because it simulates battlefield conditions insofar as it has the elements of violent exercise and excitement as well as trauma.

The blood and tissue studies of rats which had been shocked by the drum method will be reported in other papers.[7,8]

This report is concerned with results obtained with the Noble-Collip method of producing shock in the Sprague-Dawley strain of albino rats, in a strain of cancer-resistant rats, and in adrenal enucleated rats of the Sprague-Dawley strain.

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Wisconsin.

[1] Noble, R. L., and Collip, J. B., *Quart. J. Exp. Physiol. and Cog. Med. Sci.*, 1942, **31**, 187.

[2] Noble, R. L., and Collip, J. B., *ibid.*, 201.

[3] Noble, R. L., *Am. J. Physiol.*, 1942, **138**, 346.

[4] Zahl, P. A., Hutner, S. H., and Cooper, F. S., *J. Pharm. and Exp. Therap.*, 1943, **77**, 143.

[5] Chambers, R. W., Zweifach, B. W., and Lowenstein, B. E., *Am. J. Physiol.*, 1943, **139**, 123.

[6] Clarke, A. P. W., and Cleghorn, R. A., *Endocrinol.*, 1942, **31**, 597.

[7] McShan, W. H., Potter, V. R., Goldman, A., Shipley, E. G., and Meyer, R. K., *Am. J. Physiol.*, 1945, **145**, 93.

[8] LePage, G. A., unpublished data.

Materials and Methods. The apparatus used for producing shock was like that described by Noble and Collip except that the drums had a diameter of 16 inches as recommended by the authors.[1] Each of the drums had two baffles and the drums were set to rotate at 40 r.p.m. Metal screen was used to replace the glass in the doors so as to provide better ventilation.

Adult male albino rats of the Sprague-Dawley strain, weighing 250-300 g were used. These rats had been in the laboratory 5 days or longer before being used in any of the experiments. The cancer-resistant strain of rats were those maintained by Dr. M. F. Guyer of the Zoology Department and we are indebted to him for supplying us with them. Males weighing 250-300 g were used.

The adrenal-enucleated rats were Sprague-Dawley rats in which the adrenal capsule had been opened and the cortex and medulla removed by applying light pressure to the capsule. The animals were maintained on 1% NaCl as drinking water for 10 days until the adrenal remnant had regenerated sufficiently to maintain the animals. The rats weighed 125 to 175 g at the time of adrenal enucleation. They were used for the experiment five to six weeks later, at which time the weight ranged from 260 to 360 g. It was obvious that their appetites had been normal and that normal growth had ensued since the average weights were approximately doubled.

Rats were considered to survive if they lived more than 24 hours after tumbling in the drums. To eliminate the possibility of protection from jumping over the baffles the forepaws were fastened together with adhesive tape before the rats were placed in the drums.

Results and Discussion. The animals dying from drum trauma showed essentially the same changes in appearance as described by Noble and Collip.[1] These included extreme vascular dilatation in the intestine and its mesentery, reddening of the muscles of the abdominal wall, frequent hemorrhage into the lumen of the intestine and petechiæ in the intestinal walls. Lungs sometimes showed either petechiæ or marked congestion. The heart contained little blood and it was thick and viscid. Rarely did an animal show any subcutaneous, cranial, or muscular hemorrhage. Teeth and claws were frequently broken and the feet were often slightly bruised. The liver was engorged and sometimes small breaks and tears occurred. No broken or torn places were ever observed in the spleen. Both adrenals and kidneys often showed congestion.

TABLE I.
Survival of Rats to Drum Shock.

No. of rats	Time,* min.	% survival
14	4	100.0
44	8	65.6
42	10	71.4
125	12	47.2
63	13	9.5
16	15	4.3

* Drum revolved 40 times per minute.

The data in Table I show the results obtained by use of 304 male rats of the Sprague-Dawley strain. The per cent of animals surviving is less in almost all cases than was obtained by Noble and Collip at comparable periods of rotation. These results together with ours on conditioning[11] indicate that the Sprague-Dawley strain of rats is not as resistant to trauma of this type as are the rats of the strain used by Noble and Collip.

Table II summarizes the results of testing the effect of varying one factor at a time on survival of groups of rats. The first factor which was modified was that of taping all paws in contrast to taping only the front paws as was done regularly when producing Noble-Collip shock. Slightly better survival was obtained when both pairs of paws were taped together. The survival was 42.9% as compared to 21.5% when only the front paws were taped.

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[9] Freeman, N. E., Freedman, H., and Miller, C. C., *Am. J. Physiol.*, 1941, **131**, 545.

[10] Bulbring, E., and Burn, J. H., *J. Physiol.*, 1942, **101**, 289.

[11] Shipley, E. G., Meyer, R. K., and McShan, W. H., unpublished data.

TABLE II.
Alteration in Resistance of Rats to Drum Shock by Various Factors.

No. of rats	Time run,* min.	Factor modified	% survival
14	12	Taping paws—all taped	42.9
14	12	,, ,, fore paws taped	21.5
19	12	Bled one week previously	5.1
8	12	Without abdominal girdle	0.0
14	12	With ,, ,,	50.0
6	12	Adrenal enucleation	0.0
6	12	Intact adrenals	16.7
24	10	Adrenal enucleation	8.3
12	10	Intact adrenals	50.0
20	12	Cancer resistant	20.0
10	10	,, ,,	30.0

* Drum revolved 40 times per minute.

To determine the effect of protection to the abdomen on survival, 2 groups of rats were run simultaneously in the Noble-Collip drums for 12 minutes. One group was without protection and the second group was protected by having a strip of heavy muslin wrapped snugly around the abdomen of the rat and fastened in place with adhesive tape. The strips were wide enough to cover the space between the fore and hind legs and was long enough to make 4 or 5 thicknesses when in place. Some degree of protection against the development of shock was afforded by girdling the abdomen but this protection was not complete, as is indicated by the data in Table II. Chambers, Zweifach, and Lowenstein[5] found that protection of the abdomen during tumbling prevented the development of a shock-like state in rats subjected to rotation up to 1000 revolutions. Fifty per cent of our rats with abdominal protection survived while none of the controls survived.

Four groups of rats which had survived bleeding to produce hemorrhagic shock were subjected, one week later, to 12 minutes of Noble-Collip shock to see if protection had developed as a consequence of the bleeding. Only 1 rat out of 19 survived the Noble-Collip shock. Comparing the 5.2% survival in these rats with the 47.2% survival in normal rats, it is obvious that a decreased rather than an increased resistance resulted.

The adrenal-enucleated rats were run simultaneously with control rats. Ten or 12-minute periods of tumbling were used. None of the adrenal-enucleated and only 1 control survived 12 minutes. At 10 minutes 8.33% of 24 adrenal-enucleated and 50% of 12 unconditioned rats survived. All of the adrenal-enucleated rats were autopsied and the regenerated adrenals were removed to determine whether regeneration had occurred. In all cases regeneration of the cortex had occurred. Thus it would seem that loss of the adrenal medulla or perhaps inadequate regeneration of cortical tissue had decreased the percentage of survival. Freeman, Freedman, and Miller[7] produced a shock-like state by the infusion of adrenaline. Bülbring and Burn[10] found that small amounts of adrenaline augment transmission in sympathetic ganglia and adrenaline in large amounts depresses transmission. The authors concluded that sudden liberation of large amounts of adrenaline was more likely to produce permanent circulatory damage than small amounts. If liberation of adrenaline contributes to the production of shock, then the removal of the adrenal medulla should increase the percentage survival in shocked animals, which have been subjected to overwhelming sensory stimuli. However, the results of our experiments do not support this conclusion but it is to be emphasized that the cortex was also largely removed, leaving remnants under the capsule for regeneration. Several investigations have shown that adrenalectomized animals are more susceptible to all types of shock than are normal animals.[2,12,13,14] Numerous investigators have tried the effects of adrenal-cortical extracts as prophylactic and as therapeutic agents in shock produced by various means in both normal and adrenalectomized animals. Some of them have found the adrenal cortical hormones of benefit[15–17] and others found no beneficial effects from use of these hormones.[18–25] In general it may be said prophylaxis with adrenal cortical extracts was found to be of more benefit than therapeusis, and that a greater increase in survival was found in treated adrenalectomized than in treated intact animals. The findings of these authors together with our results in adrenal-enucleated rats suggest that a minimum quantity of adrenal cortical hormones are needed to protect an animal against trauma and that increasing the amount of the extracts is of no further benefit, whereas a decrease in quantity is detrimental.

As a cancer resistant strain of rats was readily available it became of interest to determine whether resistance to cancer might be linked with resistance to trauma. Included in Table II are the results obtained from subjecting 30 adult male rats of the cancer-resistant strain to graded degrees of trauma in the Noble-Collip apparatus. The survival at 12 minutes and 10 minutes were respectively 20% and 30%. These results do not seem to show any marked variations from those obtained with the Sprague-Dawley strain, but the percentages of survival are slightly lower.

Summary. 1. Varying degrees of shock can be produced in rats by subjecting them to various periods of trauma in Noble-Collip drums. The method appears to be suitable for use where graded and reproducible degrees of trauma are desired.

2. The percentages of survival were: for 5 minutes of tumbling, 100%; for 8 minutes, 65.6%; for 10 minutes, 71.4%; for 12 minutes, 47.2%; for 13 minutes, 9.5%; and for 15 minutes, 4.3%.

3. The Sprague-Dawley strain and a cancer-resistant strain of rats were found to be less resistant to trauma of the Noble-Collip type than were rats used by other investigators.

4. Survival of previous hemorrhage did not increase resistance to Noble-Collip trauma, but appeared to decrease resistance to some extent.

5. Adrenal enucleation decreased the resistance of rats to Noble-Collip trauma although regeneration of cortical tissue had been sufficient to support normal growth and to maintain the rats in a healthy state.

6. Protection to the abdomen and taping of the paws gave only limited protection to the rats of the Sprague-Dawley strain subjected to Noble-Collip shock.

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[12] Swingle, W. W., and Parkins, W. M., *Am. J. Physiol.*, 1935, **111**, 426.

[13] Swingle, W. W., Remington, H. W., Collings, J. W., and Parkins, W. M., *Am. J. Physiol.*, 1941, **132**, 249.

[14] Hechter, O., Krohn, L., and Harris, J., *Endocrinol.*, 1942, **31**, 439.

[15] Katz, L. N., Killian, S. T., Asher, R., and Perlow, S., *Am. J. Physiol.*, 1942, **137**, 79.

[16] Weil, P. G., Rose, B., and Browne, J. S. L., *Canad. Med. Assn. J.*, 1940, **43**, 8.

[17] Shleser, I. H., and Asher, R., *Am. J. Physiol.*, 1942, **138**, 1.

[18] Besser, E. L., *Arch. Surg.*, 1941, **43**, 249.

[19] Fine, J., Fischmann, J., and Frank, H. A., *Surg.*, 1942, **12**, 1.

[20] Huizenga, K. A., Brofman, B. L., and Wiggers, C. J., *J. Pharm. and Exp. Therap.*, 1943, **78**, 193.

[21] Ingle, D. J., *Am. J. Physiol.*, 1943, **139**, 460.

[22] Keating, F. R., Jr., Power, M. H., and Rynearson, E. H., *Current Research in Anesth. and Analg.*, 1942, **21**, 207.

[23] Koster, H., and Kasman, L. P., *Arch. Surg.*, 1942, **45**, 272.

[24] Swingle, W. W., Overman, R. R., Remington, J. W., Kleinberg, W., and Eversole, W. J., *Am. J. Physiol.*, 1943, **139**, 481.

[25] Wigger, C. J., *Univ. Hosp. Bull. Ann Arbor*, 1943, **9**, 61.

15182

Failure of Homozygous Embryo Skin to Prevent Growth of Autogenous Tumor-Grafts in the Rat.*

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A question of considerable interest was brought to my attention on the subject of induced resistance. The question was whether induced resistance can be produced by normal tissues. This concerns the relationship between the immunizing agent and the genetic antecedent of the host. For example, a number of investigators[1-11] were able to induce a resistant state to malignant growths including leukemia in hybrid animals by injecting normal homologous tissues, such as liver, spleen, blood, and embryo skin. On the other hand, similar results were not obtained[12-14] when pure lines of inbred strains of animals were used. From the two sets of observations, it was concluded that the degree of induced resistance which can be produced depends upon the genetic relationship between the host and the immunizing agent, and that no resistance to tumor growth can be produced either with normal or with malignant tissues in animals of a pure inbred line.

However, the author has reported[15] that there is a possibility of inducing immunity in animals of a pure line to a tumor which originated in the same strain, provided the tumor grafts had been previously attenuated *in vitro* with specific doses of X-rays.

It was of interest to investigate whether or not the same phenomenon could be demonstrated by the use of normal embryo skin of the same pure inbred strain of rats, for it has been shown[6,8,11,14] that embryo skin is the most effective agent among normal tissues in this respect.

The technic employed here was similar to that employed by previous investigators[14] and consisted of the following: the skin of rat embryos was removed under strictly aseptic conditions, cut with sharp scissors into minute particles and a concentrated suspension prepared in a small amount of 0.85% saline solution. Portions of 0.3 cc of this suspension were injected subcutaneously in the right side of a rat which had been previously shaved and cleaned with alcohol. Twelve days later, the left sides of the same rats were implanted by means of a trocar with grafts weighing about 3-4 mg of the reticulum cell type lymphosarcoma which originated in the same strain. As controls 6 rats of the same strain were implanted with grafts of the same tumor. All the rats of these experiments, both experimental and control, were males. The same criteria were used in the evaluation of the

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[1] Bashford, E., *Brit. Med. J.*, 1906, **11**, 209; *3rd Scient. Rep. Imp. Cancer Fund*, Vol. 1904-1908, p. 322.

[2] Shoene, G., *Med. Woch.*, 1906, **53**, 2517.

[3] Levine, I., PROC. SOC. EXP. BIOL. AND MED., 1910, **7**, 107.

[4] Lambert, R. A., PROC. SOC. EXP. BIOL. AND MED., 1911-12, **9**, 18.

[5] Woglom, W. H., *J. Exp. Med.*, Jan., 1910, p. 29.

[6] Haaland, M., *Proc. Royal Soc. London, Series B*, 1909-1910, **82**, 293.

[7] Rhoades, C. P., and Miller, D. K., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 817.

[8] Kamekura, R., *Z. f. Immunitatsforschg.*, 1925, **49**, 49.

[9] Gardner, R. E., *Am. J. Hygiene*, 1931, **13**, 649.

[10] McDowell, E. C., *Proc. Nat. Acad. Sci.*, 1935, **21**, 507.

[11] Sheever, F. S., *Cancer Res.*, 1941, **1**, 23.

[12] McDowell, E. C., Taylor, M. J., and Potter, J. S., *Proc. Nat. Acad. Sci.*, 1935, **21**, 507.

[13] Barrett, M. R., *J. Nat. Cancer Inst.*, 1940, **1**, 387.

[14] Eisen, M. J., and Woglom, Wm. H., *Cancer Res.*, 1941, **1**, 629.

[15] Goldfeder, Anna, PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 104.

TABLE I.
Tumor Takes in Rats Inoculated with Embryo Skin (of the same strain) Prior to Tumor Implantation.

No. of rats	Latent period, days	Initial tumor size, mm	Maximum tumor size, mm	Remarks
1	10	8x7x6	35x18x14	Died 24 days following implantation of tumor.
2	10	5x4x4	30x16x14	Died 24 days following tumor transplantation.
3	10	6x5x4	33x23x20	" 26 " " " "
4	10	6x6x5	28x20x19	" " " " " "
5	10	7x6x5	36x25x18	" " " " " "
6	12	6x6x4	35x24x20	" 28 " " " "
7	12	4x4x3	38x25x24	" " " " " "
8	12	6x4x4	28x25x29	Killed for tumor transplantation.
9	12	5x4x4	29x21x18	All these rats died within 30 to 40 days following implantation of the tumor.
10	14	6x5x4	32x26x22	
11	14	4x4x3	28x24x16	
12	14	5x5x4	30x25x20	
13	14	6x4x4	28x22x18	
14	14	6x5x4	32x25x19	
15		No growth occurred		This rat was resistant to the primary and to subsequent tumor grafts.

results as in the previous experiments,[15] namely: the percentage of takes, the latent period of the takes, and the initial and maximum tumor size.

In Table I are recorded the observations made on the rats treated with homologous embryo skin prior to tumor grafting and in Table II those of controls. A comparative analysis of the data recorded in these tables reveals the following: Of the 15 experimental rats in Table I 14 developed tumors following a latent period from 10 to 14 days, while among the 6 control rats recorded in Table II all developed tumors following a latent period of 12 days. Judged by the initial and maximum tumor sizes of both experimental and control experiments, the tumors developed at approximately the same rate until the death of the animals.

No significance can be attributed to the fact that one of the 15 rats treated with embryo skin was refractory to the tumor grafts, or to the extended latent period of 14 days among some of these rats (Table I) instead of 10 to 12 days occurring among controls (Table II) in regard to the immunizing effect of the embryo skin. Such single instances were repeatedly observed among control animals of previous experiments. This observation holds particularly true where larger numbers of animals are being used. Rat 15, which proved to be refractory to the first tumor graft, was found likewise to be refractory to subsequent tumor implants. Such a phenomenon has been repeatedly observed among rats of previous experiments. If the first graft fails to produce a tumor, the animal remains refractory to subsequent tumor grafts.

It seems safe to conclude, from the ob-

TABLE II.
Tumor Takes in Control Rats of the Same Strain.[‡]

No. of rats	Latent period, days[*]	Initial tumor size, mm	Max. tumor size, mm[†]
1	10	5x5x4	31x18x15
2	10	7x5x4	35x22x17
3	10	6x4x4	34x19x15
4	12	5x5x4	28x20x16
5	12	5x4x4	34x30x19
6	12	7x5x4	38x29x22

[*] Latent period designates time elapsing between implantation of tumor grafts and first appearance of a detectable tumor.

[†] Maximum tumor size reached at death of animal.

[‡] All rats died within 25 to 40 days following implantation of tumor grafts.

servations recorded in the Tables I and II, that inoculations of embryo skin into rats of the same genetic constitution, fail to elicit a resistant state to grafts of a tumor autogenous to the strain. This observation is in accord with findings of previous investigators who likewise failed to elicit immunity in a pure line of animals with embryo skin of the same genetic line.[14]

Discussion. There is no intention here to discuss generally the genetic principles involved in the problem of induced resistance to malignant growth. As indicated in the previous communication[15] the results obtained are specifically concerned with the reticulum cell type lymphosarcoma, originating in a pure line of rats of Dr. Bagg. It was suggested that the type of tumor may be responsible for the specific immune action in an inbred strain of rats. The results obtained from the experiment described in the present paper brought further evidence, in this case, that the induced resistance is specific to the type of tumor and independent of the genetic inter-relation between the host and the immunizing agent, inasmuch as embryo skin of the same strain proved to be ineffective in eliciting the same phenomenon.

On the other hand the results of the present paper are in full agreement with those obtained by other investigators, showing that no resistance to tumor grafts can be elicited in animals of a pure line with embryo skin of the same strain.

Summary. Rats of a pure inbred line treated with embryo skin of the same strain failed to produce a resistant state to grafts of a tumor autogenous to the strain.

15183

Shock Produced by the Application of Tourniquets to the Hind Limbs of Rats.*

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The tourniquet method has been accepted as a valid means of producing experimental shock and numerous investigators have used some variety of tourniquets on laboratory animals, including albino rats. Some investigators have used rubber bands,[1,2] cord tied tightly or wrapped in numerous turns about the leg,[3-8] and compression from clamp devices to cut off the circulation to a limb.[9,10] We were interested in standardizing the tourniquet method so that graded and reproducible degrees of trauma could be effected. Animals shocked by this method were to be used in making biochemical studies for comparison with similar studies[11,12] on rats shocked by the

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Wisconsin.

[1] Allen, F. M., *Am. J. Surg.*, 1943, **60**, 335.

[2] Allen, F. M., *Arch. Phys. Ther.*, 1943, **24**, 327.

[3] Ingle, D. J., *Am. J. Physiol.*, 1943, **139**, 460.

[4] Katzenstein, R., Mylon, E., and Winternitz, M. C., *Am. J. Physiol.*, 1943, **139**, 307.

[5] Kleinberg, W., Remington, J. W., Eversole, W. J., Overman, R. R., and Swingle, W. W., *Am. J. Physiol.*, 1943, **140**, 197.

[6] Mylon, E., Winternitz, M. C., and de Suto-Nagy, G. J., *Am. J. Physiol.*, 1943, **139**, 313.

[7] Mylon, E., Winternitz, M. C., Katzenstein, R., and de Suto-Nagy, G. J., *Am. J. Physiol.*, 1942, **137**, 280.

[8] Swingle, W. W., Overman, R. R., Remington, J. W., Kleinberg, W., and Eversole, W. J., *Am. J. Physiol.*, 1943, **139**, 481.

[9] Green, H. N., Camb, M. A., and Sheff, M. SC., *Lancet*, 1943, **11**, 147.

[10] Haist, R. E., and Hamilton, J. I., *J. Physiol.*, 1944, **102**, 471.

[11] LePage, G. A., unpublished data.

[12] McShan, W. H., Potter, V. R., Goldman, A., Shipley, E. G., and Meyer, R. K., *Am. J. Physiol.*, 1945, **145**, 93.

Noble-Collip[13] technic.

The purpose of this paper is to report: (1) the method developed for producing graded tourniquet shock, (2) the effect of re-application of tourniquets on the percentage survival in rats, and (3) the hemoconcentration which occurred in tourniquet-shocked rats.

Materials and Methods. The animals used for this study were adult male rats obtained from Sprague-Dawley, Inc. The animals were kept in our laboratory for at least 5 days before being used, at which time they weighed 250-325 g. They were maintained on the regular stock diet and tap water *ad libitum.*

The tourniquets used were designed so that a set screw could be adjusted against a metal bar in such a way as to tighten a loop of cord slipped over the leg of the rat. The metal portions of the tourniquets were of brass and the cord was ordinary fish line. The tourniquets were applied to both hind legs as high on the thighs as possible and tightened until the screws could no longer be turned by the normal amount of force applied by the fingers. When this was accomplished the muscles of the legs were rigid and the circulation was occluded as shown by cyanosis. The tourniquets were left in position for varying periods of time, depending upon the degree of shock desired.

The rats were anesthetized by Nembutal (Abbott Laboratories) given intraperitoneally just before the tourniquets were adjusted. The usual dose required was 0.2 cc. During the time the tourniquets were in place the animals were given additional amounts (0.05-0.10 cc) of Nembutal as needed to keep them in a semi-conscious state, but the anesthetic was not given after the tourniquets were removed.

As an indication of the course of hemoconcentration occurring after the removal of the tourniquets, hemoglobin determinations were made on tourniquet-shocked rats by the method of Evelyn[14] for oxyhemoglobin. Blood for these studies was obtained by cutting off the tip of the tail.

Results and Discussion. Survival after tourniquet shock: The percentages of animals surviving application of tourniquets for various periods of time are summarized in Table I.

TABLE I.
Survival of Rats Subjected to Tourniquets for Varying Periods of Time.

No. of rats	Tourniquet applied		% survival
	hr	min	
7	3	30	100
8	3	35	75
15	3	40	47
12	3	45	33.3
33	4	0	3.0
4	4	15	0
6	4	30	0
4	8	0	0*

* None of these rats survived more than 3 hours after removal of the tourniquets.

It can be seen that the number of animals surviving decreased as the period of tourniquet application increased. The exact length of the survival period was not determined for every animal that died since some animals died during the night. The usual period of survival was 6 to 18 hours after removal of the tourniquets, but there seemed to be no correlation between the length of the period that the tourniquets were in place and the length of the survival period in those animals which died, except that when the tourniquets were applied for 8 hours the rats all died within 3 hours after removal of the tourniquets (Table I). The usual course of events after tourniquet removal was a period of approximately 4 or 5 hours in which the animal seemed to be in good condition, in spite of the fact that the legs showed increasing edema. The circulation was restored soon after the release of the tourniquets except in an occasional rat in which the vessels to the limb were permanently occluded as indicated by the continued cyanosis of the limb and the absence of edema. In some cases cyanosis remained yet edema developed. This could be due to venous occlusion or loss of whole blood into the traumatized area. During the first 1 or 2 hours after release the legs showed the greatest increase in size, but they continued to become gradually larger during the course of the first 5 or 6 hours. During that period fluid was lost into the tissue adjacent to the tourni-

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[13] Noble, R. L., and Collip, J. B., *Quart. J. Exp. Physiol. and Cog. Med. Sci.*, 1941, **31**, 201.

[14] Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.

TABLE III.
Hemoglobin Concentration in Rats in Tourniquet Shock.*

No. of rats	1† Hb–before tq. removal	2 Hb–after tq. removal	3 Hb–increase	4 Hb–last sample	5 Hb–increase	Survival +/—	Hour of death
	%	%	%	%	%		
1	84.0	111.5	27.5	111.5	27.5	—	7
2	85.0	137.2	52.2	137.2	52.2	—	7
3	92.0	126.4	34.4	126.4	34.4	—	8
4	79.0	117.3	38.3	117.3	38.3	—	4
5	98.1	130.1	32.0	121.8	23.7	Sacrificed	5
6	81.6	126.4	44.8	118.6	37.0	”	5
Avg	86.6	124.8	38.2	122.1	35.5		

* 100% hemoglobin equals 15.6 grams per 100 cc.

† The values in column 3 were obtained by subtracting the values in column 1 from those in 2; the values in column 5 by subtracting those in 1, from the ones in 4.

quet-traumatized tissue, and into the genital region, particularly the scrotum. After these first hours changes in gross size could not be detected by observation. Haist and Hamilton[10] found that the swelling of the limb reached its maximum during the second hour after removal of the clamps, but in their rats the clamps had been left on overnight (12-15 hrs) and the average survival time was only 3 hours 12 minutes. It might be expected that maximum edema would be reached more rapidly after longer periods of tourniquets.

Hemoconcentration in tourniquet shock: Hemoconcentration is usually an accompaniment of shock and it was of interest to follow the course and extent of hemoconcentrations in normal rats during the period following tourniquet removal when shock was developing. Hemoconcentration may be determined by measurement of hematocrit, by increase of specific gravity, by hemoglobin determinations and by red cell counts. In our laboratory hemoglobin determination was the most feasible method to use as an index of hemoconcentration.

It is known that splenic contraction occurs in shock, and that it contributes to hemoconcentration. Taylor and Page,[15] using dogs, found that "the splenic contribution to changes in the hematocrit index occurs during the first one or two hours of the experiment." The authors state that splenectomy abolishes the early sharp rise of the hematocrit index seen in shock. On the basis of these studies it is probable that the early increase in hemoglobin observed after the removal of tourniquets can be ascribed only in part to fluid loss. This fact in no sense invalidates the use of hemoglobin determinations as indications of hemoconcentration since we were interested in relative rises in hemoconcentration extending over a number of hours.

Hemoglobin determinations were made on rats which had tourniquets in place for 4 hours. The first determinations were made at the time of the removal of the tourniquets, and each hour thereafter for periods as long as 7 hours. Two of the animals for which data are given were sacrificed at the end of 5 hours to obtain blood for analyses to be reported in connection with other blood studies.[12] Table III is a summary of the hemoglobin values found during the period after the tourniquets were removed, and shows the highest concentration found for each of the animals during the experimental periods, that found in the last sample, and the increases of each of these over the original concentration. In the shocked rats hemoconcentration developed progressively after the removal of the tourniquets until the death of the animal. Increases in hemoglobin in rats which were followed to the hour of death ranged between 27 and 52%. From these data the conclusion can be drawn that marked hemoconcentration is an accompaniment of shock produced by tourniquets and that the wide variation in degree of hemo-

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[15] Taylor, R. D., and Page, I. H., *Arch. Surg.*, 1943, **47**, 59.

TABLE II.
Survival of Rats After Reapplication of Tourniquets Following Varying Periods of Release.

No. of rats	Tourniquet applied	Tourniquet released	Tourniquet reapplied	% survival
	hr	hr	hrs	
6	4	1	Through 24	100
6	4	2	,, 24	100
6	4	3	,, 24	100
6	4	4	,, 24	100
8	4	5	,, 24	100
8	4	6	,, 24	100
8	4	7	,, 24	87.5

concentration in animals at the time of death would preclude the use of it as an indication that the irreversible stage had been reached in tourniquet shock.

Reapplication of tourniquets: Experiments were designed to find the stage at which the irreversible condition is reached in tourniquet shock, and to determine if it is the period of ischemia alone or some subsequent change that is the decisive factor in this type of shock. Adult male rats were subjected to tourniquets for 4 hours, then the tourniquets were released for periods varying from 1 to 7 hours, then replaced and left in position through 24 hours from the time of first release. The rats were lightly anesthetized with ether for the replacement of the tourniquets, but they were not further anesthetized during the period of reapplication. The tourniquets were then removed and the rats were observed for the next 24 hours to determine survival rates. The data in Table II summarize the results of this experiment and by comparison with those in Table I it can be seen that only 3% of the rats survived 4 hours of tourniquets when there was no replacement, but in the case of temporary release for 1 to 6 hours 100% of the animals survived during the following 24 hours of reapplication. The tourniquets were then released and the animals were observed for an additional 24 hours. Two animals died after the second release of the tourniquet. Death in one of these rats followed severe hemorrhage from the traumatized tissue. The rats were regularly sacrificed after 48 hours because the limbs were necrotic. After replacement of tourniquets the legs had become cyanotic and remained so after the tourniquets were released. The rats were otherwise alert and energetic. When the release period was increased to 7 hours 87.5% of the animals survived when tourniquets were reapplied. The irreversible stage of shock was probably prevented from developing by the elimination of circulation to the injured limbs and the prevention of any further loss of fluid into the injured area and the escape of metabolites from the injured tissue. Thus the animal could successfully cope with the first loss of fluid and the first of the metabolites from the injured area. Green *et al.*[9] found that after clamping both legs of rats for 7 hours removal of limbs up to 2½ hours saved all animals, but "from 2½ hours onward the survival rates steadily fell, to reach zero, on the average, at 4 hours." They further observed that the rat could withstand repeated sublethal doses of clamping totalling at least 12 out of 32 hours.

It would appear that local fluid loss to the limb after release of the tourniquets cannot be the only factor responsible for the development of irreversible shock as reapplication of the tourniquets after fluid loss has occurred prevents death. The importance of fluid loss in decreasing the ability of the animal to recuperate cannot be denied, but it is to be emphasized, as blood[12] and muscle analyses show,[11] that after tourniquet removal substances from the limb escape into the circulation. The accumulation of these metabolic breakdown products probably interferes with cellular metabolism and damage to vital organs results. The beneficial effect following reapplication of tourniquets suggests that the total quantity of the injury substances, as well as the length of time over which they act, is of importance in determining the fate of the animal.

Summary. 1. A method for producing

tourniquet shock in rats is described. The method yields graded and reproducible results.

2. The percentage survival of rats to tourniquet shock was progressively decreased as the time of tourniquet application was increased. Following 3½ hours of tourniquets 100% of the rats survived, while after 4 hours of tourniquets only 3% survived.

3. Rats which had tourniquets applied for 4 hours with periods of release for 1 to 6 hours, followed by a second application of tourniquets, showed 100% in contrast to 3% survival for those without replacement, while 87.5% survived when the tourniquets were replaced after 7 hours.

4. The degree of hemoconcentration developing in rats in tourniquet shock was approximated by hemoglobin determinations. Increases in hemoglobin ranged between 27 and 52%.

15184

Inactivation of Estrone by Liver. Assay Method *In vivo* for Dietary Hepatic Injury in Rats.*

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Pellets of estrone implanted in the spleen will not induce estrus in ovariectomized rats fed a normal diet.[1] Estrone draining from the pellet through the splenic vein into the liver is inactivated by the normal functioning liver parenchyma, either through direct chemical destruction[1] or through a closed "hepato-intestinal cycle."[2] Failure of the liver to inactivate estrogen has been demonstrated by Biskind and Biskind[1] in rats maintained on a diet deficient in the vitamin B complex. Estrus in such animals appeared within 1 to 3 weeks and was interpreted as an indication of impaired liver function. Such functional change resulting from avitaminosis is in its etiology and pathogenesis different from the specific hepatic injury (necrosis and cirrhosis) produced in rats by the administration of a synthetic diet low in lipotropic factors (methionine and choline),[3] but containing all known vitamins including the members of the vitamin B complex. On the other hand, however, it has been shown[4] that with regard to inactivation of estrogen by the liver, rations deficient in lipotropic factors will have the same effect as rations free of vitamin B complex. In spite of this analogy only the disturbance elicited by rations deficient in lipotropic factors with its characteristic pathological manifestations is considered the true dietary hepatic injury.[3]

In previous experiments[4] the onset of functional disability of the liver, measured by the failure of inactivation of estrone, became evident in rats fed a diet low in lipotropic factors after an interval when severe histological changes are known to make their appearance. This interval was much longer than that observed in rats fed a vitamin B-free diet.[1,4] Whereas the impaired inactivation of estrone in rats kept on a vitamin B-free diet could be reversed and normalized by supplements of vitamin B complex,[1,4] large doses of yeast were needed to achieve the same result in rats

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* Aided by a grant from Wyeth, Incorporated, Philadelphia.

[1] Biskind, M. S., and Biskind, G. R., *Endocrinol.*, 1942, **31**, 109. Here also literature.

[2] Cantarow, A., Paschkis, K. E., Rakoff, A. E., and Hansen, L. P., *Endocrinol.*, 1943, **33**, 309.

[3] György, P., and Goldblatt, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 492; Webster, G., *J. Clin. Invest.*, 1941, **20**, 440; Blumberg, H., and McCollum, E. V., *Science*, 1941, **93**, 598; Lillie, R. D., Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, 1941, **56**, 1255.

[4] Shipley, R. A., and György, P., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 52.

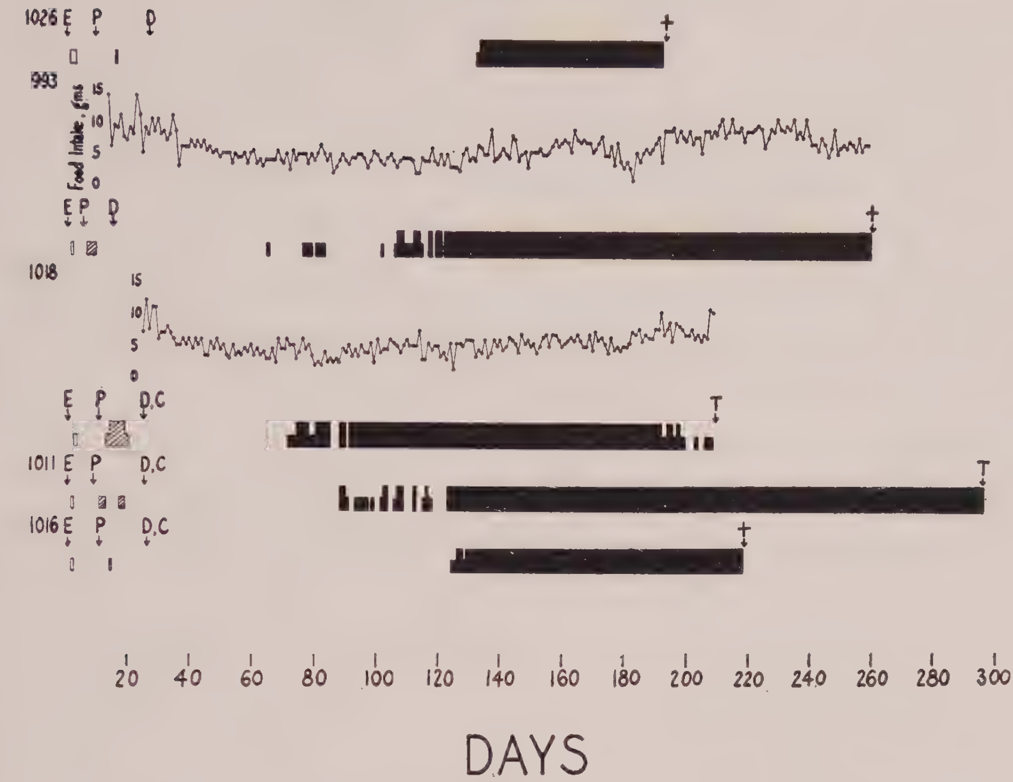

FIG. 1.

Series of rats receiving a diet low in proteins and high in fat. (5 of 9 rats in Group A). The blocked areas represent estrus. Partial estrus is depicted by a reduction in height of the block. Symbols are as follows: E—Test dose of estrone, P—Insertion of pellet, D—Diet started, C—Supplement of cystine, T—Experiment terminated. +, death of animal. The rats were castrated at zero time.

fed a diet low in lipotropic factors.

Reversal of the estrus reaction by the administration of large doses of yeast indicates that curative measures for dietary hepatic injury may be adequately assessed in the living animal.

Yeast as a curative factor in dietary hepatic injury is in good accord with previous findings obtained in prophylactic experiments of long duration (150 days). Addition of yeast to the basal experimental diet prevented the production of hepatic injury as controlled by macroscopic and microscopic examinations of the liver.[5] However, yeast is not the best and most characteristic representative of the lipotropic substances. Given as sole source of protein and not—as in our previous studies—as supplement to a diet with a low casein content as source of protein, yeast may even support dietary hepatic injury, especially necrosis.[6] Furthermore, yeast is rich in vitamins of the B group and in many other cell constituents. Thus, curative effect exerted by yeast has to be considered too complex to permit detailed analysis. Judging from prophylactic studies based on pathological findings in the dead animal a curative effect similar to that of yeast might be logically anticipated from simple substances such as methionine or methionine-containing proteins, for example casein or lactalbumin. In contrast, even manifold increased vitamin B supple-

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[5] György, P., and Goldblatt, H., *J. Exp. Med.*, 1942, **75**, 355. Also György, P., *Am. J. Clin. Path.*, 1944, **14**, 67, with bibliography.

[6] Hock, A., and Fink, H., *Z. f. physiol. Chem.*, 1943, **278**, 136, and 1943, **279**, 187; Himsworth, H. P., and Glynn, L. E., *Lancet*, 1944, **1**, 457; Glynn, L. E., and Himsworth, H. P., *J. Path. and Bact.*, 1944, **56**, 297.

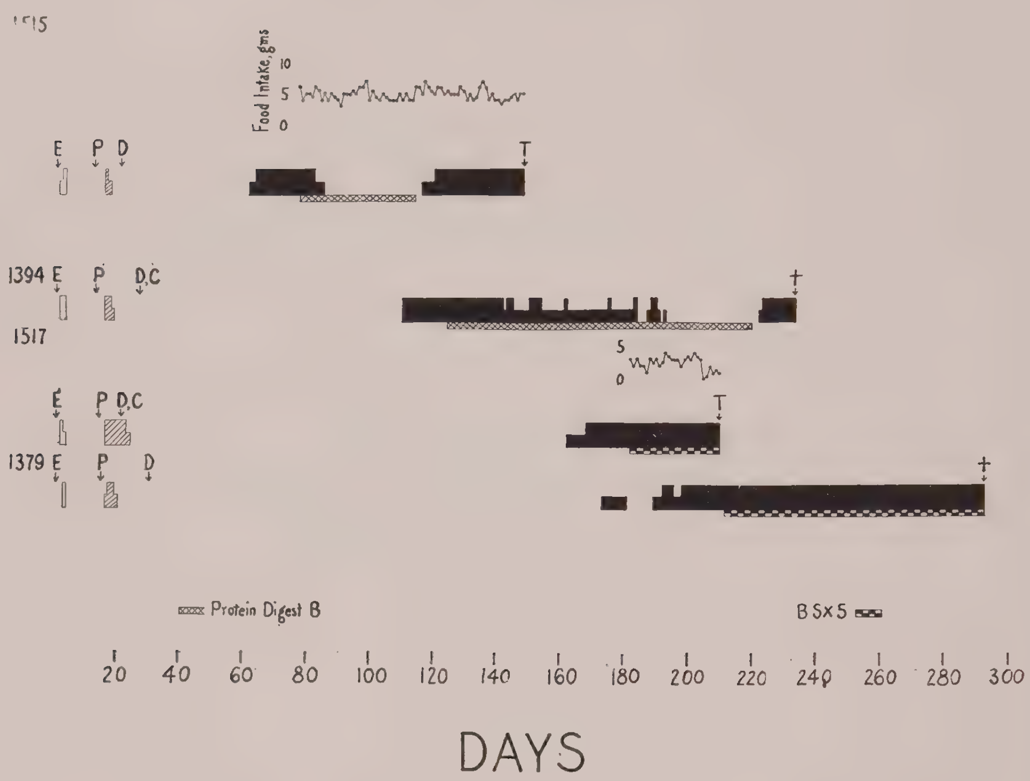

FIG. 2.

The effect of protein digest—lactalbumin digest (in 2 of 5 rats in Group D) and of fivefold vitamin B supplements (in 2 of 13 rats in Group E). The symbols have the same meaning as in Fig. 1.

ments should prove to be devoid of any curative effect.

The following experiments deal mainly with these special questions. They were undertaken to produce supporting evidence for the usefulness of the method of estrus reaction in the study of experimental dietary hepatic injury in the living animal.

Methods. Rats used were of 3 separate strains: 1. Sprague-Dawley (65 rats), 2. Wistar (9 rats), and 3. a strain obtained from a local dealer (17 rats). Animals were selected with a weight of 150-230 g, the majority weighing approximately 200 g. The preparation of the animals for the experiments, including surgical technic, was the same in the previous study.[4] The experimental diet was slightly modified, as follows: Casein (SMACO) 8, Crisco 38 (or, in some experiments, Crisco 30 and peanut oil 8), sucrose 50, salt mixture 4. The vitamin supplements were given separately: (a) Solution of B vitamins (1 cc daily) containing thiamine 20 γ, pyridoxine 20 γ, riboflavin 25 γ, Ca-pantothenate 100 γ. (b) Vitamins A and D in form of Ol. percomorph., 3 drops weekly. (c) Vitamin E as special supplement was only used in the experiments with the local and Wistar strains (3 mg in the form of natural concentrate[†] once a week). In the remaining experiments the vitamin E requirement was covered solely by the basal diet with its high content of Crisco, a fat rich in vitamin E.[‡] More than half of the experimental animals received as a further separate supplement 50 mg of *l*-cystine daily. The purpose of this supplement was the acceleration and aggravation of cirrhotic changes in the liver.[5]

[†] Kindly furnished by Distillation Products, Inc., Rochester, N.Y.

[‡] According to a personal communication of Dr. K. E. Mason (Rochester), 5% of Crisco in the diet represents the minimal protective level.

Results. The appearance of estrus showed great variations. It is remarkable that 35 out of a total of 91 experimental animals remained in anestrus for the whole duration of observation, up to 150 days or more. Two rats showed only temporary or irregular estrus. In the remaining group of 54 rats regular estrus was observed 39 to 195 days, with a calculated average of 86 days after the animals were put on the basal experimental diet. In several instances estrus when first noticed showed some fluctuation before going into solidly sustained complete estrus (Fig. 1, rats 993 and 1011).

On the assumption that normal liver "inactivates" estrone, lack or retarded appearance of estrus should indicate absence or delayed production of hepatic injury with its characteristic pathological attributes. Such behavior would be at variance with the reactivity of normal rats[5] and could only be explained by the presence of estrone pellets in the spleen preceded by ovariectomy in the present experimental group of animals.[§]

Rats showing the appearance of continuous estrus were kept under observation for 2-4 weeks without any change in the experimental conditions. At the end of this preliminary period the animals were divided in 5 subgroups (A to E). Subgroup A was kept as control on the unchanged original regime. Rats in B received *dl*-methionine[||] in daily doses of 50 mg, in C a digest of casein (Amigen) 1 g daily, in D a digest of lactalbumin (Lactamin)[||] 1 g daily. In subgroup E the daily basal vitamin supplements were increased fivefold. In instances when cystine was given before the appearance of regular estrus, it was discontinued as soon as treatment with lipotropic factors (methionine or protein digests) started.

Once established, full estrus persisted throughout the total period of observation in Group A (9 animals), in which the original experimental conditions remained unchanged, and in Group E (13 rats) in which the animals received a fivefold increase in the amount of basal vitamin B supplements. The solid curve of full estrus in such animals (See Fig. 1 and 2 with representative examples) served also as control for experiments in which estrus has disappeared under the influence of special curative measures. Of course, even without special treatment estrus, when artificially supported by a pellet of estrone implanted in the spleen, cannot last permanently and must cease as soon as the pellet is completely absorbed or becomes tightly encapsulated (Rat No. 1018 in Fig. 1). This last possibility has to be borne in mind in cases where estrus was seemingly suppressed after application of special curative measures. In order to avoid false conclusions a positive result should not be considered as such unless with discontinued therapy estrus reappears, thus proving the presence of properly functioning estrone pellets.

This special experimental procedure was fully observed in subgroups B, C, and D in which the therapeutic effect of methionine (16 animals), casein-digest (11 animals), and lactalbumin-digest (5 animals) was investigated. The results have shown unequivocally that methionine (Fig. 3) as well as the 2 protein-digests (representative examples for lactalbumin in Fig. 2) exerted a definite therapeutic effect which manifested itself in complete or at least in partial suppression of estrus. Interruption of treatment was followed in due course by reappearance of full estrus. The time required for the suppression of estrus by means of supplements of lipotropic factors showed considerable variation, ranging from a few days to 10 weeks, with an average of 31 days.

In a number of rats (in all subgroups) daily food intake was measured. No correlation could be found between appearance or disappearance of estrus and the curve of daily food intake.

Discussion. The assumption submitted previously[4] that impairment of estrone inactivation by the liver observed in rats on a cirrhosis-producing diet resulted from the specific dietary hepatic injury, is well borne out by the present studies. Fivefold increase in the amount of daily vitamin B supplements

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[§] This special problem will be discussed in a separate communication. Preliminary report appeared in *Federation Proceed.*, 1945, **4**, 155.

[||] Kindly furnished by Wyeth, Incorporated.

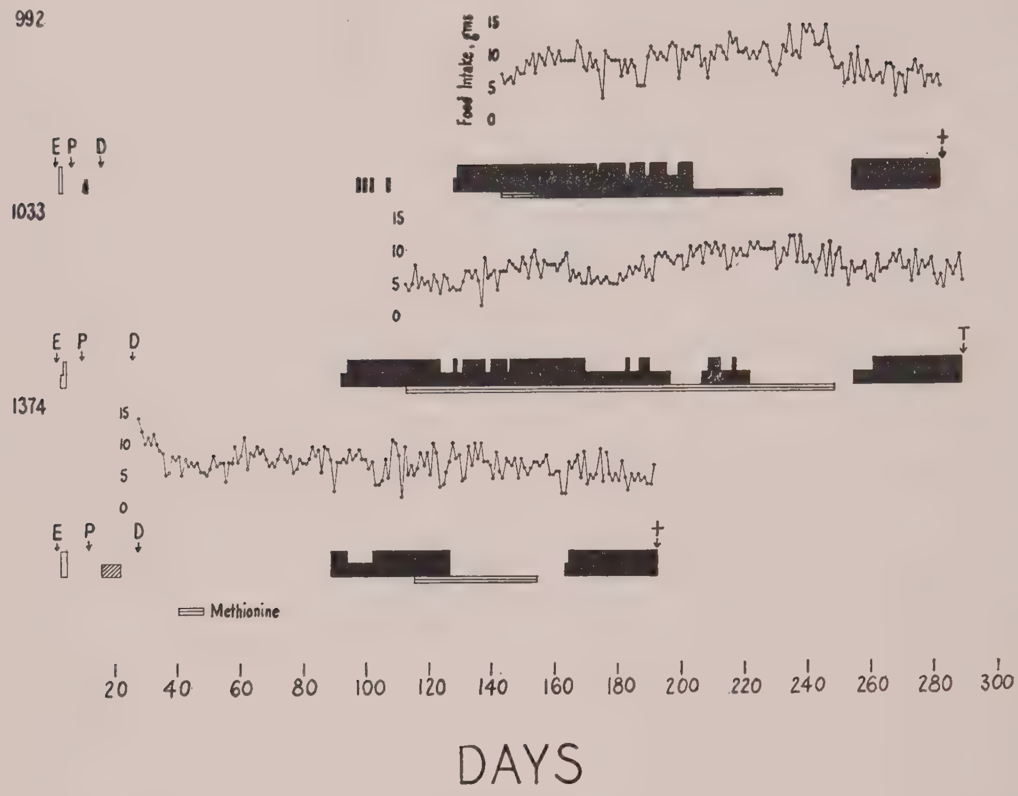

FIG. 3.

The effect of methionine (in 3 of 16 rats in Group B). The symbols have the same meaning as in Fig. 1 and Fig. 2.

had no effect on estrus appearing in this group of rats. Thus it can be denied that the liver injury produced by cirrhosis-producing diet has led to impairment of estrone inactivation solely by increasing the need for the B vitamins. Such relative vitamin B deficiency would have been the counterpart of the similar effect of absolute lack of B vitamins.[1]

With the demonstration of the therapeutic effect exerted by lipotropic factors such as methionine or methionine-containing protein digests (casein and lactalbumin) the specific character of the impairment of estrone inactivation in this group of rats is well substantiated. The same lipotropic factors play a leading part in the prevention of dietary hepatic injury, proven by macroscopic and microscopic evidence.[5] The present observations should arouse special interest for two reasons: 1. They were made on living animals, and 2. They indicate strongly the possibility of therapeutic reversal of the underlying hepatic changes, in contrast to past routine studies of prophylactic nature. Inasmuch as treatment was initiated soon after the functional hepatic disturbance became apparent, it is improbable that major fibrotic changes in the liver could have been developed before therapy was started. In consequence, the restored suppression of estrus should not be construed as an outward sign of completely restored architecture. Morphological cure may be deduced only from direct histological examination. On the other hand the methods of estrone inactivation could become useful in the evaluation of possible therapeutic factors on living animals suffering from dietary liver injury. It is conceivable to regard estrus appearing in rats fed a cirrhosis-producing diet as the equivalent of pseudo-estrus of vitamin A deficiency, based on the inability of cirrhotic livers to utilize vitamin A of the diet. This explanation, however, disregards the important fact that estrus disappeared even in cirrhotic rats as soon as the estrone pellet was completely absorbed. Lack of estrone should not influence the pseudo-estrus of vitamin A deficiency.

Summary. Impairment in ability to inactivate estrone accompanies dietary hepatic injury and can be rectified by the addition of lipotropic factors such as methionine or protein digests.

Fivefold increase of the basal vitamin B supplements was ineffective.

The author is greatly indebted to Mrs. C. K. Gilkey for technical assistance.

15185

Studies on Toxicity Complement-Fixing and Immunogenic Activity of Typhus-Infected Yolk Sacs.

VINCENT GROUPÉ AND RICHARD DONOVICK.* (Introduced by Geoffrey Rake.)

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The use of the yolk sac of the developing chick embryo for cultivating typhus rickettsiæ[1] led to the development of vaccines prepared from infected yolk sacs by phenol precipitation[2] and ether extraction[3,4] and to the demonstration of a labile toxic substance in yolk sac cultures of typhus rickettsiæ which was lethal for mice and which was associated with the rickettsiæ themselves.[5,6]

It has been shown that both the complement-fixing activity and the rickettsial content of typhus vaccines were related to the time of harvest of the constituent yolk sacs with reference to the peak of embryo deaths.[7] However, it was also observed that the presence of numerous rickettsiæ did not necessarily indicate toxicity[6] and that the antibody response of guinea pigs, commonly used as a measure of antigenicity, is subject to considerable variations.[8] It was of interest, therefore, to compare complement-fixing activity with the toxicity of yolk sac suspensions and with the antigenicity and rickettsial content of typhus vaccines.

Methods. Eggs in the 7th day of embryonic development were inoculated into the yolk sac with the Breinl strain of epidemic typhus according to the method of Cox.[1] The eggs were candled daily and all yolk sacs harvested were frozen immediately and stored at −70°C until used. All vaccines were prepared by the ether extraction method[4] using a standard procedure (*cf.* [7]).

Complement-fixation tests were done according to the method of Bengtson.[9] Vaccines were titrated with 2 units of hyperimmune guinea pig serum and the titer was recorded as the highest dilution of vaccine showing complete (4+) fixation.

Toxicity titrations were performed as follows: 0.5 ml of serial 2-fold dilutions of the various yolk sac suspensions was injected into

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[*] On temporary leave from the Division of Microbiology, The Squibb Institute for Medical Research.

[1] Cox, H. R., *Pub. Health Rep.*, 1938, **53**, 2241.

[2] Cox, H. R., *Science,* 1941, **94**, 399.

[3] Connaught Laboratories, Toronto, Ontario, War Project Med. 8, November 13, 1942 (N.R.C. Canada), Memorandum No. 3.

[4] Topping, N. H., National Institute of Health, Washington, D.C., 1945, Bull. No. 183.

[5] Gildemeister, E., and Haagen, E., *Deut. Med. Wchnschr.*, 1940, **66**, 878.

[6] Bengtson, I. A., Topping, N. H., and Henderson, R. G., Nat. Inst. of Health, Washington, D.C., 1945, Bull. 183.

[7] Groupé, V., Nigg, C., and MacFarlane, J. O., *J. Immunol.*, in press.

[8] Donovick, R., Farrell, M., and Smith, F., *J. Bact.*, 1945, **50**, 241.

[9] Bengtson, J. A., *Pub. Health Rep.*, 1941, **56**, 649.

TABLE I.
Comparison of Toxicity with the Complement-Fixing Activity of Infected Yolk Sac Suspensions.*

Interval between inoculation and harvest	No. of yolk sacs represented	Complement fixation test suspension diluted:			Toxicity titration suspension diluted:			
		1–16	1–32	1–64	1–2	1–4	1–8	1–16
L-5	15	0	0	0	0/3†	0/4	0/4	
L-6	15	3+	1+	±	4/4	4/4	0/4	0/4
L-7	15	4+	2+	±		4/4	1/4	0/4
L-8	15	4+	4+	2+			4/4	0/4

* 10% tissue suspensions by weight.
† No. of mice dead/ Total No. of mice.
L-5 = Embryos living 5 days after inoculation.

the tail vein of mice.† The number of deaths was recorded 18 hours after inoculation.

The approximate rickettsial content of a vaccine was estimated by microscopic examination of films of vaccine made with a standard loop and stained by Macchiavello's technic (*c.f.* [7]).

Antigenicity was measured by immunizing† mice as follows: Method A: Each of 30 mice was given one intraperitoneal inoculation of 0.2 ml of undiluted vaccine. Method B: Groups of 10 mice each were given one intraperitoneal inoculation of 0.5 ml of vaccine diluted 1-5, 1-25, and 1-125 respectively. Fourteen days after vaccination all vaccinated mice were challenged with 0.5 ml of toxic substance representing 2-4 LD_{50} injected into the tail vein. The results were recorded 18 hours after challenge.

Experimental. Comparison of toxicity with complement-fixing activity of infected yolk sac suspensions. Inasmuch as both the rickettsial content and complement-fixing activity of vaccines were highest when the constituent yolk sacs were harvested from embryos still living at the peak of embryo deaths,[7] yolk sacs were harvested only from living embryos to prevent loss of toxicity following death of the embryo. In comparing toxicity with complement-fixing activity yolk sacs were harvested from 15 living embryos on the 5th, 6th, 7th, and 8th day after inoculation respectively. Each pool of yolk sacs thus obtained was frozen immediately and stored at –70°C. Approximately one week later 10% untreated tissue suspensions in saline were prepared from each pool of yolk sacs and each suspension was titrated for complement-fixing activity and for toxicity in mice. It will be seen from Table I that the complement-fixing titer of yolk sac suspensions increased from less than 1-16 in the case of suspension L-5, prepared from embryos harvested 5 days after inoculation, to 1-32 in the case of suspension L-8, prepared from embryos harvested 8 days after inoculation. When the same suspensions were titrated for the presence of toxic substance by intravenous injection of mice suspension L-5 diluted 1-2 was not lethal for mice whereas suspension L-8 diluted 1-8 was lethal for all mice inoculated. It is thus evident that the complement-fixing activity and toxicity of untreated saline suspensions of infected yolk sacs harvested from living embryos rose together.

Comparison of antigenicity with complement-fixing activity and rickettsial content of typhus vaccines. The complement-fixing activity, rickettsial content and antigenicity of typhus vaccines prepared from yolk sacs harvested before and at the peak of embryo deaths were compared and the results are presented in Table II. It was found that the complement-fixing titer increased from less than 1-8 in the case of vaccine D-5, prepared from embryos dead 5 days after inoculation, to 1-64 in the case of vaccine L-7, prepared from embryos still living 7 days after inoculation and that the rickettsial content of the vaccines increased with the complement-fixing activity. When mice were immunized by Method A only 6 of 28 (21.4%) of the mice immunized with vaccine D-5 were protected whereas 17 of 27 (62.9%) of the mice immunized with vaccine D-6 and 24 of 28 (85.7%)

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† Young albino Swiss mice (Webster strain) weighing 12-13 g were used throughout these experiments.

TABLE II.
Comparison of Complement-Fixing Activity, Rickettsial Content and Antigenicity of Vaccines Prepared from Embryos Harvested Before and at the Peak of Embryo Deaths.

		Vaccines prepared from yolk sacs harvested from embryos:				
		D-5	D-6	D-7	L-7	
No. of yolk sacs included in vaccine		90	102	146	65	
Rickettsial content		±	+	++	++++	
Complement fixation test Vaccine diluted:	1-8	3+				
	1-16	1+	4+	4+	4+	
	1-32	±	3+	4+	4+	
	1-64	0	2+	3+	4+	
	1-128	0	0	0	1	
Antigenicity for mice						
Method A—0.2 ml undiluted vaccine inoculated I.P.						
Vaccinated group						
Total No. of mice		28	27		28	
No. protected		6/28*	17/27	n.t.	24/28	
% protected		21.4	62.9		85.7	
Control group Challenge dose diluted:	1-2					0/16*
	1-4					10/15
Method B—0.5 ml of serial dilutions of vaccine inoculated I.P.						
Vaccinated group Vaccine diluted:	1-5	0/9	4/9	n.t.	7/10	
	1-25	0/10	0/10		6/10	
	1-125	0/10	0/10		0/10	
Control group Challenge dose diluted:	1-2					0/10
	1-4					8/10

D-5 Dead 5 days after inoculation.
L-7 Living 7 days after inoculation.
+ Five rickettsiae per field.
++++ Over 100 rickettsiae per field.
n.t. Not tested.
4+ Complete fixation of complement.
0 No fixation of complement.
* No. of mice surviving/Total No. of mice.
I.P. Intraperitoneally.

of the mice immunized with vaccine L-7 were protected. When mice were immunized by Method B vaccine D-5 diluted 1-5 did not protect any of the 9 vaccinated mice whereas vaccine D-6 diluted 1-5 protected 4 of 9 vaccinated mice, and vaccine L-7 diluted 1-25 protected 6 of 10 vaccinated mice. In addition to the data presented, in 7 additional experiments the complement-fixing activity of the vaccine likewise followed the antigenicity of the vaccine.

Discussion. It is clear from the data presented that complement-fixing activity followed not only the rickettsial content and antigenicity for mice of vaccines prepared in an identical manner but also the toxicity of yolk sac suspensions when care was taken to preserve toxicity. However, the factors responsible for toxicity and complement-fixing activity are not identical since toxicity is destroyed by formalin and ether[6] while complement-fixing activity is not. Furthermore, the complement-fixing activity of a given yolk sac suspension or vaccine represents not only the rickettsiæ themselves but also a soluble antigen capable of immunizing guinea pigs.[10] Nevertheless, when yolk sac suspensions or vaccines were prepared as described from embryos harvested on consecutive days an increase in complement-fixing activity was always accompanied by an increase in toxicity or antigenicity.

Summary. Under the conditions described, the toxicity of untreated yolk suspensions and the antigenicity of typhus vaccines prepared from embryos harvested on consecutive days rose together with the complement-fixing activity of the same antigens.

The authors wish to acknowledge the technical assistance of Mr. Jack O. Davis.

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[10] Topping, N. H., and Shear, M. J., *Pub. Health Rep.*, 1944, **59**, 1671.

15186

Synergistic Action of Nicotinamide Upon Penicillin.

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In the course of some studies dealing with the mechanism of penicillin action, it was observed that relatively high concentrations of nicotinamide appeared to increase measurably the effectiveness of penicillin against the Oxford strain of *Staphylococcus aureus.* Since, of the B vitamins, only para-aminobenzoic acid has been shown to affect penicillin activity,[1,2] this synergistic action has been investigated in more detail.

Experimental Methods. In testing the effectiveness of compounds under investigation for their synergistic action, it was found most convenient to compare the concentrations of penicillin necessary to produce half-maximum inhibition of the test organisms in the presence and absence of the agent under study. The organisms being investigated were grown in a modified proteose-peptone medium, with added glucose and yeast extract. Short (5-7 hour) growth periods were used. Growth levels were estimated turbidimetrically, using a Klett-Summerson photoelectric colorimeter.

In most of the experiments, Lederle penicillin (potency 400 Oxford units per mg) was used. Crystalline sodium penicillin G was obtained through the courtesy of Merck and Co. Eastman Kodak Company nicotinamide and nicotinic acid were used without purification, except as noted below.

Results. The addition of nicotinamide to the culture medium in concentrations less than about 250 γ per ml has no apparent effect upon the inhibition of *Staphylococcus aureus* by penicillin. Beyond this point, however, significant effects are observed. Representative data are shown in Fig. 1. It is evident that the concentration of penicillin necessary for half-maximum inhibition decreases with increasing concentration of the amide. Concentrations of nicotinamide greater than 1.7 mg/ml were not studied.

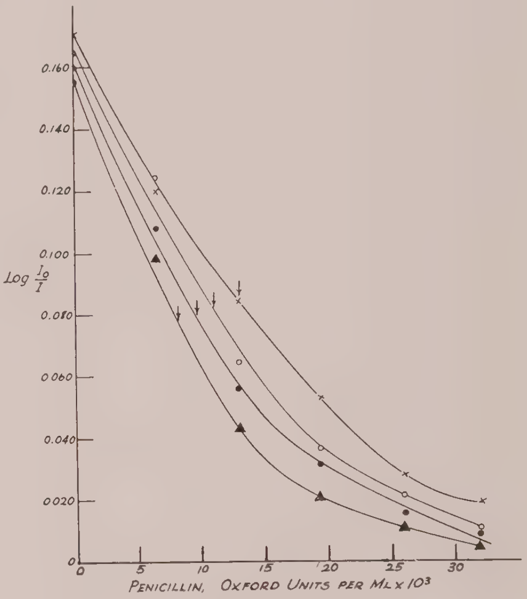

FIG. 1.

Effect of increasing concentrations of nicotinamide upon penicillin inhibition of *Staphylococcus aureus* (Oxford). The concentrations of nicotinamide, in mg/ml, are as follows: ×—0.00; ○—0.40; ●—0.80; ▲—1.60. The vertical arrows indicate half-maximum inhibition points.

Despite the fact that nicotinic acid is frequently interchangeable with nicotinamide in bacterial nutrition,[3] it was found that sodium nicotinate was unable to duplicate the action of nicotinamide when used in comparable concentrations.

The relatively high nicotinamide levels necessary to produce observable synergism suggested the possibility that the effects noted were in reality due to an impurity in the preparation used, although two different lots of nicotinamide, obtained from the same manufacturer at different times, gave identical

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[1] Ungar, J., *Nature,* 1943, **152**, 245.

[2] Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 184.

[3] Peterson, W. H., and Peterson, M. S., *Bact. Rev.*, 1945, **9**, 49.

results. That this was not the case was shown by the fact that purified nicotinamide, recrystallized from either dioxane or alcoholic benzene, gave results which were indistinguishable from those obtained with the cruder preparation. Likewise, identical results were had when the impure penicillin preparations were replaced by crystalline penicillin at comparable concentrations.

The possibility that the effects observed were due to a slow reaction of penicillin with nicotinamide to form a product superior in antibacterial activity to penicillin itself was next investigated. On the basis of this hypothesis, the high nicotinamide concentrations required are necessary to force the reaction measurably toward completion in the relatively short periods involved. Accordingly, mixtures of nicotinamide and penicillin (including mole ratios from 4:1 to 4,000:1) were incubated at 37°. After 18 hours, the solutions were diluted so that the residual nicotinamide concentrations were less than 20 γ per ml, and inhibition curves determined. These were compared with curves for a solution of penicillin alone which had been treated similarly. No enhancement of penicillin action could be observed under these conditions.

In an effort to determine the effect of nicotinamide upon penicillin inhibition of other organisms, experiments similar to those described above were performed using *Streptococcus pyogenes* C-203, *E. coli,* and two other strains of *Staphylococcus aureus*. Penicillin inhibition of *S. pyogenes* and *E. coli* was unaffected by the presence of even large amounts of nicotinamide. However, the other strains of *Staphylococcus aureus* studied responded to combinations of penicillin and nicotinamide in a manner qualitatively and quantitatively analogous to the response of the Oxford staphylococcus. In the presence of 1.5 mg of nicotinamide per ml, the concentration of penicillin required for half-maximum inhibition of the latter organism is approximately 0.7 that required when no nicotinamide is present. Ratios for the other strains studied were 0.64 and 0.67 respectively. It should be noted that these 3 strains had significantly different penicillin sensitivities.

It is well known that penicillin is most effective under conditions favoring rapid growth of the organism, and the theory that penicillin acts primarily upon actively multiplying cells is supported by a good deal of evidence.[4] It seems unlikely, however, that the phenomenon under discussion can be explained by an increased rate of cellular multiplication. Although detailed studies of the effect of nicotinamide upon the rate of growth of the staphylococcus have not been made, a single experiment indicated that a slight *decrease* in rate occurred upon using concentrations of nicotinamide comparable to those indicated above.

Summary. High concentrations of nicotinamide exert a synergistic effect upon penicillin inhibition of *Staphylococcus aureus*. Nicotinic acid was inactive in this respect. No evidence for a reaction between nicotinamide and penicillin could be found. Although the effect of nicotinamide appeared to be general for the 3 strains of staphylococcus tested, no effect could be demonstrated upon either *Streptococcus pyogenes* or *E. coli.*

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[4] Chain, E., and Duthie, E. S., *Lancet*, 1945, **1**, 652.

15187 P

Studies on the Action of Penicillin. IV. Development of Penicillin Resistance by Gonococcus.*

C. Phillip Miller and Marjorie Bohnhoff.

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Among the several recent reports on the development of penicillin resistance by various microörganisms, only that by Bahn, Ackerman, and Carpenter[1] concerns gonococcus. They were able to enhance the penicillin tolerance of one of 5 strains of gonococcus sufficiently after 32 weeks to permit growth in blood broth containing 2 units of penicillin per ml. The other strains responded less readily, one scarcely at all. Lankford[2] found that among 203 freshly isolated strains of gonococcus none was resistant to more than 0.02 units of penicillin per ml.

As gonococci grow more readily on solid media than in broth, the following experiments were made by surface cultivation on agar containing known concentrations of penicillin.

Methods. Strains of gonococci were transferred daily to freshly prepared blood agar plates[3] containing increasing concentrations of penicillin.[†] Each transfer was made to 3 or 4 plates containing graded increments of penicillin and taken from the plate which had developed confluent growth during 24 hours incubation. This procedure was followed because it was found that enhancement of penicillin tolerance proceeded more rapidly when transfer was made from media containing the highest concentration of penicillin which permitted moderately abundant growth rather than from higher concentrations which produced only a few colonies.

The penicillin agar was prepared each day just before use because its growth inhibiting potency was found to diminish even in the icebox.

The results with one strain are plotted in Fig. 1. The penicillin tolerance; that is, the ability to multiply on penicillin agar, increased rapidly at first and reached its limit at 7.7 units per ml. Just before that occurred and at a time when the average daily increment had become small, a second series of transfers was begun by subcultivation onto penicillin-free agar. Thereafter, this second series was transferred back and forth from penicillin-containing to penicillin-free agar. As will be seen in the chart, this practice resulted in further and more rapid enhancement of penicillin tolerance which reached a level of 21 units per ml. The only detectable difference in this second series of transfers was more luxuriant growth on penicillin-free agar which provided a greater number of viable gonococci to be carried back onto penicillin agar. These results indicate that enhancement of penicillin resistance proceeds most rapidly when vigorous multiplication is maintained.

To test the validity of the foregoing explanation, a strain of gonococcus was repeatedly subjected to prolonged exposure to concentrations of penicillin which inhibited reproduction. To 2 cc volumes of blood broth containing varying concentrations of penicillin, approximately 5×10^6 gonococci per ml were

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* A portion of the work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

[1] Bahn, Jeanne M., Ackerman, Helen, and Carpenter, Charles M., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 21.

[2] Lankford, Charles E., *Am. J. Syph. Gon. and Ven. Dis.*, 1945, **29**, 56.

[3] The medium is described by Miller, C. Phillip, Moeller, Velma, and Bohnhoff, Marjorie, Proc. Soc. Exp. Biol. and Med., 1945, **58**, 143.

[†] The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutics and Other Agents of the National Research Council.

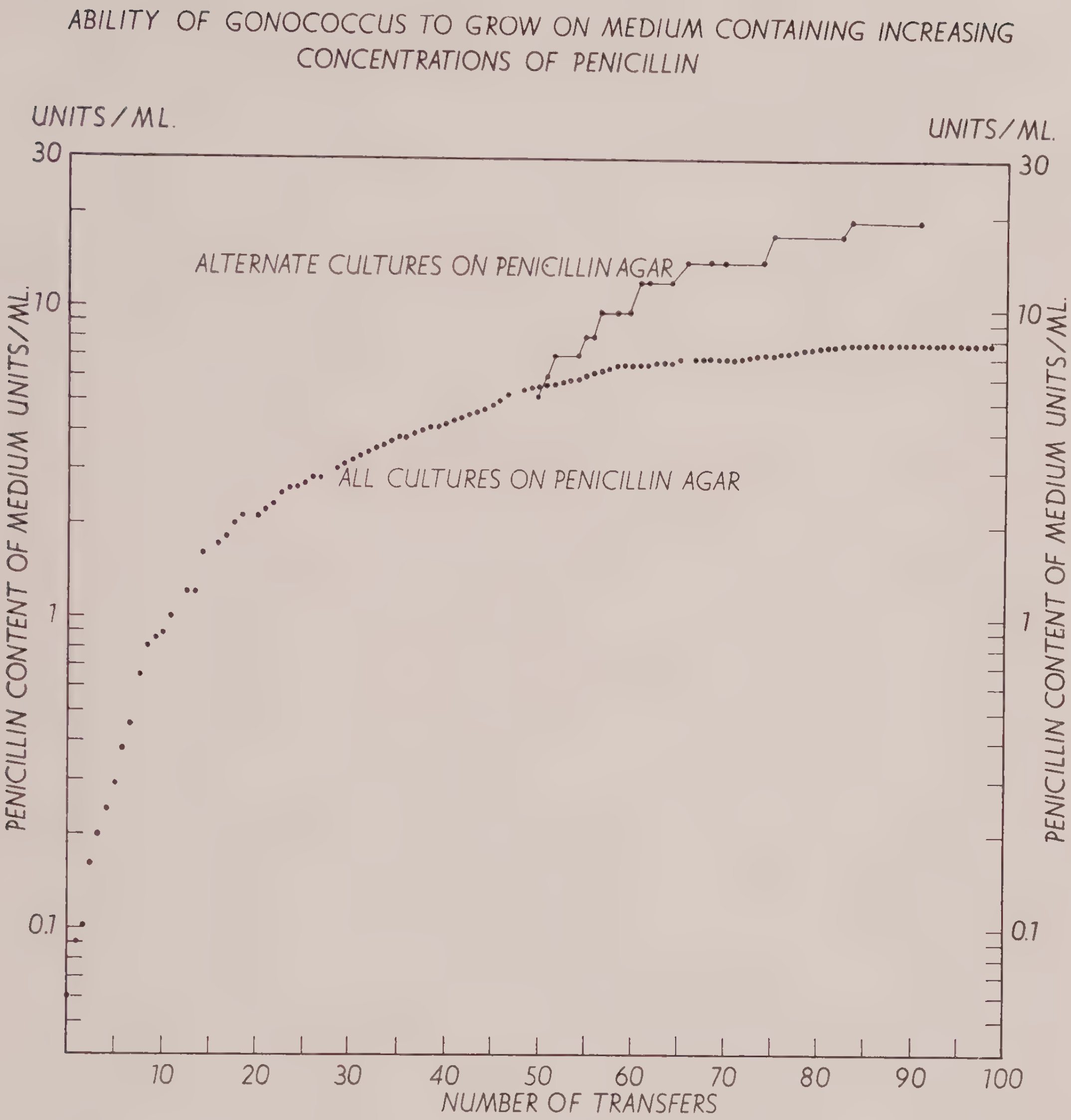

Fig. 1.

mixed in Wassermann tubes, incubated overnight, then centrifuged at high speed for one-half hour. The number of viable gonococci surviving was determined by culturing the centrifuged sediment onto freshly poured blood agar plates which were incubated in a candle jar and examined at 24 and 48 hours. After the first 24 hours of incubation, colonies developing from organisms surviving the highest concentration of penicillin were grown on penicillin-free agar and used for the next suspension in penicillin broth.

After 13 successive suspensions in penicillin-broth, the number of viable gonococci surviving exposure to each concentration of penicillin had not increased (beyond the limits of the error of the method) and indicate that the resistance of the strain had not been appreciably increased.

At the time of the first penicillin broth suspension and again after the 9th, the strain was tested for its ability to multiply on penicillin agar. The change was insignificant, being 0.04 and 0.05 units per ml, respectively.

The reliability of the method was checked by subjecting to this treatment organisms which had developed ability to multiply on agar containing different concentrations of penicillin. Multiplication always occurred in broth containing penicillin in the concentration just below that of the agar from which the inocula were taken.

Penicillin-fast strains of gonococcus showed distinct morphological changes, which were retained during subsequent cultivation on penicillin-free agar. They will be described in a subsequent report.

Penicillin resistance developed *in vitro* was not permanently retained during prolonged cultivation on penicillin-free agar. Its rate of loss has not yet been accurately determined.

Summary. Penicillin-fastness developed most rapidly in gonococci under conditions which permitted the greatest number of viable microörganisms to be transferred to a higher concentration of penicillin at each transfer. One strain of gonococcus acquired the ability to grow on media containing 21 units per ml. No appreciable increase in penicillin tolerance resulted from repeated exposure to bacteriostatic concentrations of penicillin.

15188 P

Studies on Action of Penicillin. V. Virulence of Penicillin Resistant Strains of Meningococcus.

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Several reports have appeared on the development of penicillin resistance *in vitro* and *in vivo* by staphylococci[1,2] and pneumococci,[3] but they do not agree on its effect on virulence.

Methods. The sensitivity of meningococci to the bacteriostatic action of penicillin* was determined by transferring them from routine media onto a series of blood agar plates[4] containing known concentrations of penicillin and incubating them in a candle jar. The highest concentration permitting the development of any colonies was considered the limit of that strain's resistance to penicillin. Increased resistance to penicillin was developed by the method described for gonococci.[5] Virulence was determined by inoculating mice intraperitoneally with mucin suspensions of meningococci.[6] Susceptibility to the therapeutic action of penicillin was estimated by infecting mice with 10,000-100,000 M.L.D. of meningococci and injecting them subcutaneously 3 and 6 hours thereafter with appropriate doses of penicillin.

Results. The natural resistance to penicillin was determined on 96 strains of meningococci and found to vary from 0.1 to 0.5 units per ml. Seven strains were selected at random for enhancement of penicillin resistance and were subcultured daily on media containing increasing concentrations of penicillin. The resistance of all 7 strains increased at about

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[1] McKee, Clara M., and Houck, Carol L., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 33.

[2] Spink, Wesley W., Hall, Wendell H., and Ferris, Viola, *J. A. M. A.*, 1945, **128**, 555.

[3] Schmidt, L. H., and Sesler, Clara L., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 353.

* The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

[4] The medium is described by Miller, C. Phillip, Moeller, Velma, and Bohnhoff, Marjorie, PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 143.

[5] Miller, C. Phillip, and Bohnhoff, Marjorie, PROC. SOC. EXP. BIOL. AND MED. (preceding communication).

[6] Miller, C. Phillip, and Castles, Ruth, *J. Inf. Dis.*, 1936, **58**, 263.

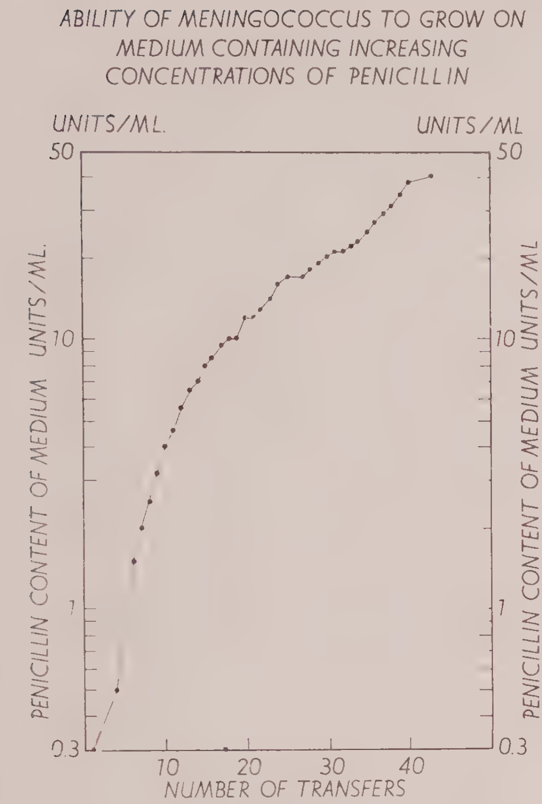

Fig. 1.

the same rate. None was carried to its limit. The one strain (No. 274) which was carried farthest became contaminated at a time when it was growing on media containing 41 units per ml. The results with this strain are shown in Fig. 1.

The virulence of all 7 strains was determined after they had acquired varying degrees of penicillin resistance up to 5 units per ml and all were found to have lost very little or none of their original virulence for mice.

Table I summarizes observations on the *in vitro* and *in vivo* action of penicillin on strain 274 up to the level at which it lost virulence. These include determinations of virulence and susceptibility to therapeutic action in the infected mouse. The slight diminution of virulence which occurred as penicillin resistance developed was no more than commonly occurs during repeated subcultivation on artificial media. But when the strain had acquired resistance to 18 units per ml virulence was lost completely and could not be restored by mouse passage. The strain had also acquired unusual physical properties although it still retained its ability to ferment glucose and maltose.

TABLE I.
Changes in Virulence and in Susceptibility to Treatment by Penicillin During the Development of Penicillin Resistance by Meningococcus.

Penicillin resistance units/ml	Virulence*	Smallest protective dose of penicillin† units
0.3	maximal	20-40
5	high	400
10	,,	600
14	,,	600
18	none	not done

* maximal—M.L.D. of 1-10 meningococci
high—M.L.D. of 10-100 meningococci.
none—M.L.D. of 10^9 meningococci.

† Smallest dose of penicillin which successfully protected mice infected with 100,000 of the meningococci described in the other columns.

The table also shows that as the strain increased its tolerance *in vitro*, the test infection (always produced by inoculating the same number of microörganisms) required larger doses of penicillin for successful treatment. Mice infected with the strain when it was resistant to 14 units per ml required 600 units of penicillin for its control, a dose 15-30 times the number which was effective before the strain had begun to acquire penicillin-resistance.

Summary. None of 96 strains of meningococci was found to be naturally resistant to penicillin *in vitro*. Seven strains all acquired penicillin resistance readily and at about the same rate during repeated subcultivation on agar containing increasing concentrations of penicillin. The strains remained virulent for mice as penicillin resistance increased, except 1 strain which lost virulence at 18 units per ml. Infections produced with penicillin-resistant strains required very large doses of penicillin for their control.

15189

Antibody Response of Swine to Vaccination with Formolized Swine Influenza Virus Adsorbed on Alum.*

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The antibody response of swine to vaccination with formolized influenza virus suspended in saline solution is characterized in part[1,2,3] both by a swift increase in antibody titer and by a subsequent rapid decline in the titer to low levels. A similar transient effect of influenza virus vaccines has been observed in man.[4] Because of this rapid loss of circulating antibody, various substances have been combined with the inactivated virus to alter the adsorptive and immunological processes for the purpose of prolonging the period of high antibody titer. Friedewald[5,6] reported excellent results in mice, rabbits, and ferrets by the use of killed acid-fast bacilli, paraffin oil, and "Falba" with formalin-inactivated influenza virus A (PR8 strain), a mixture which caused much local reaction. Salk[7] found that influenza vaccine adsorbed on calcium phosphate produced better and longer-lasting protection in mice than vaccine in saline solution. In rabbits and guinea pigs sterile abscesses frequently occur[8] at the site of injection of formolized influenza virus precipitated with calcium phosphate.[9] Bodily, Corey and Eaton[10] employed alum for concentrating the influenza virus, and in limited tests in man observed no essential difference between the antibody response to alum-adsorbed vaccine and that to the vaccine prepared[11] with salt solution. In view of the considerable advantages in the use of alum[12,13] with diphtheria and tetanus toxins, the question has been investigated further in quantitative studies on the antibody response of swine to known amounts of formalin-inactivated swine influenza virus adsorbed on alum. The results obtained are reported in the present paper.

Materials and Methods. The stock vaccine, prepared on 5/15/44 and stored at 2-8°C, was identical with that used in a previous study[3] and contained 2.0 mg of virus per ml, 0.05% formalin and 1/50,000 phenyl mer-

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[†] Member, Commission on Epidemiological Survey, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army.

[‡] Consultant to Secretary of War and Member, Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army.

[1] McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *Science*, 1945, **101**, 544.

[2] McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1945, **51**, 65.

[3] McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 152.

[4] Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1942, **75**, 495.

[5] Friedewald, W. F., *Science*, 1944, **99**, 453.

[6] Friedewald, W. F., *J. Exp. Med.*, 1944, **80**, 477.

[7] Salk, J. E., *Science*, 1945, **101**, 122.

[8] McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., unpublished data.

[9] Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 165.

[10] Bodily, H. L., Corey, M., and Eaton, M. D., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 165.

[11] Hirst, G. K., Rickard, E. R., and Whitman, L., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 129.

[12] Glenny, A. T., and Südmersen, H. J., *J. Hyg.*, 1921, **20**, 176.

[13] Glenny, A. T., Buttle, G. A. H., and Stevens, M. F., *J. Path. and Bact.*, 1931, **34**, 267.

TABLE I
Dates of Vaccination and Bleeding.

Animal group	October						November						December				January	
	16	17	23	24	30	31	6	7	13	20	21	28	4	5	11	18	2	15
1		BV*		B		B		B			B			B				
2	BV		B		BV		B		B	B			B			B		
3	BV		B		B		B		BV	B		B	B			B	B	
4												BV		B	B	B	B	B
5												BV		B	B	B	B	B

* B—Bled; V—Vaccinated.

curic borate. Three separate batches of vaccine, A, B, and C, were prepared using different amounts of alum.

Batch A was prepared on 10/6/44. To 25 ml of the stock vaccine, diluted to 100 ml with sterile Ringer solution and warmed to 30°C, 50 ml of sterile 2.2% alum§ solution were added to give the concentration of alum commonly employed for preparation of commercial alum-precipitated diphtheria toxoid. The pH was adjusted to 6.5, and a heavy, white, flocculent precipitate formed immediately. After storage of the suspension overnight at 4°C, the floccules were sedimented by centrifugation (500 times gravity), and the clear, supernatant fluid was decanted. The sediment was resuspended in 100 ml of sterile Ringer solution containing 0.05% formalin and 1/50,000 phenyl mercuric borate. Hemagglutinative tests showed a loss of less than 1% of the virus, the content of which in the preparation, from nitrogen estimated by Kjeldahl, was 0.56 mg per ml. Vaccine A thus consisted of 0.5 mg (based on the amount of stock vaccine used) of formolized swine influenza virus per ml adsorbed on 1.1% alum and suspended in Ringer-formalin-phenyl mercuric borate solution.

B and C were prepared on 11/27/44 by precipitation on 0.6 and 0.3% alum, respectively. In the instance of B, 12.5 ml of 1.1% alum in formalin-phenyl mercuric borate-Ringer solution were added to 6.25 ml of the original stock vaccine. The pH was adjusted to 6.5-7.0 with 0.2 *N* NaOH; the resulting precipitate was considerably less dense than that observed in the preparation of A. Because of possible incomplete adsorption and consequent loss of virus, this precipitate was not sedimented. Instead, Ringer-formalin-phenyl mercuric borate solution was added to yield 25 ml of vaccine. For the preparation of Vaccine C, 12.5 ml of a 0.6% alum solution were used in the same manner. Kjeldahl analysis revealed the virus content of B and of C to be 0.54 and 0.53 mg per ml, respectively.

The experimental animals were swine like those previously used.[2,3] The various groups for a given experiment were chosen so that each group was comparable with the others with respect to weight and sex. The animals of the first experiment (Groups 1, 2, and 3) were somewhat larger than those usually employed,[2,3] ranging in age from 18 to 24 weeks and in weight from 61 to 138 lb, with an average of 82 lb. There were 32 females and 16 males; all of the males except 3 had been castrated. The animals for the second experiment (Groups 4 and 5) were from 16 to 20 weeks old and varied in weight from 48 to 92 lb, with an average of 67 lb. There were 20 females and 12 males; 9 of the males were castrated. A few animals of Poland-China and Duroc strains were used, but the Berkshire strain was predominant.

The animals kept in the usual way,[2,3] remained healthy except during 2 weeks of abnormally bad weather at the end of December, 1944, when many suffered from upper respiratory infections. One animal of Group 1, 3 of Group 4, and 1 of Group 5 died at this time, of what appeared to be a bacterial pneumonia. There was no evidence of influenza.

Vaccination was performed by introducing 1 ml of the vaccine subcutaneously into the

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§ Baker and Adamson Reagent, Crystalline Aluminum Potassium Sulfate, $Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$. The solution used was 5% alum on the basis of the hydrated salt.

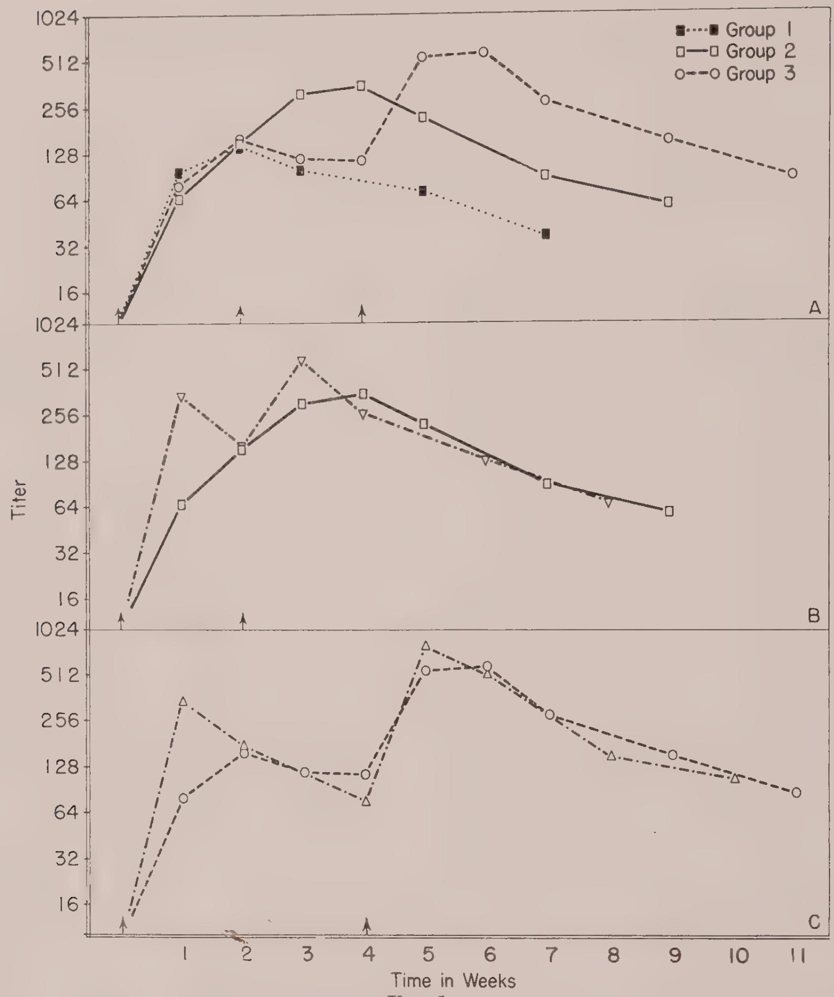

FIG. 1.

A. Antibody response of swine to vaccination with 0.5 mg of formolized swine influenza virus adsorbed on 1.1% alum. Group 1 received but one vaccination; in Groups 2 and 3 vaccination was repeated after 2 and 4 weeks, respectively.

B. Comparison of response to repeated vaccination after 2 weeks with alum-precipitated vaccine (Group 2, Fig. 1, A) with response to like vaccination with the same vaccine without alum (Group B, Fig. 2, previously reported[3]).

C. Comparison of response to repeated vaccination after 4 weeks with alum-precipitated vaccine (Group 3, Fig. 1, A) with response to like vaccination with the same vaccine without alum (Group D, Fig. 2, previously reported[3]).

right posterior axillary region where fat was avoided. Second injections were given similarly on the left side. No general reactions were observed, but all of the animals developed indurated nodules at the site of vaccination. The nodules were larger and more persistent with the larger amounts of alum. Those associated with the vaccine adsorbed on 1.1% alum were about 1.5 to 2 cm in diameter; about 70% of those initially present were still palpable after 7 weeks. The 0.6 and 0.3% alum vaccines produced nodules averaging 1 and 0.7 cm, respectively, which persisted for 7 weeks in 56 and 38% of the 2 groups of animals.

The pigs were weighed at the time of each vaccination and at the end of the experiment 7 weeks after the last vaccination; the average gain in weight was 8 lb per animal per week.

Bleeding, preparation of sera, storage, and titration of the capacity of the sera to inhibit the hemagglutinative reaction were carried out as previously described.[2,3] Sera obtained immediately before, and 1, 2, 3, 5, and 7 weeks after, vaccination were titrated twice; (1) without freezing and within 4 days of the time of bleeding, and (2) after storage in the frozen state until the completion of both experiments. The titers of the 2 sets of titrations exhibited a standard deviation of 0.47 of a 2-fold dilution, and the values given here were the geometric means of the two endpoints.

Use was made of the geometric means of the titers of the individual pigs for the reasons previously given.[2]

Results. The experiments were set up in two categories. The purpose of the initial experiment was to measure the antibody response to the vaccine adsorbed on alum and to test the response to a second vaccination with the same material after intervals of 2 and 4 weeks. Three groups of 16 pigs each were given a single dose of Vaccine A (1.1% alum), and Groups 2 and 3 were revaccinated after intervals of 2 and 4 weeks, respectively. The sequence of vaccination and bleeding is shown in Table I.

In the second experiment the relation of the response to the quantity of alum employed for adsorption was investigated. Two groups of animals, 5 and 6, were used; Group 5 was given a single dose of Vaccine B (0.6% alum) and Group 6 a single dose of Vaccine C (0.3% alum). The time relations of vaccination and bleeding in this experiment are likewise shown in Table I.

The results of experiment 1, Groups 1, 2, and 3, are shown in Fig. 1, A. The antibody titer rose sharply during the first week after the initial injection and reached the maximum at the 2-weeks interval. The rate of antibody loss in subsequent weeks is illustrated in the findings with Group 1. Repetition of vaccination of Group 2 at the 2-weeks interval resulted in a continuation of the rise initiated by the first injection to a much higher level within 1 week and maintenance of this level for another 7 days. In the instance of Group 3, a considerable diminution in antibody occurred in the interval between first and second vaccinations; nevertheless, the second vaccination of this group resulted in a rapid increase in titer, which reached a level in 1 week still higher than that of the group revaccinated after the 2-weeks interval. The high level was maintained for a period of 7 days. The rate of antibody loss after final vaccination was nearly the same in all of the groups.

In Fig. 1, B and C, are shown comparisons of the responses to repeated vaccination at the intervals of 2 (Fig. 1, B) and 4 (Fig. 1, C) weeks with vaccine adsorbed on alum (Groups 2 and 3, Fig. 1, A), and with the same vaccine without alum (Fig. 2, Groups B and D).[3] The results shown in Fig. 1, B and C, show that the overall responses to the two sorts of vaccine were strikingly similar both in degree and duration. Differences were seen only in the rate of response and in the maximum immediate response. The highest titer after the initial vaccination with alum vaccine occurred at the 2-weeks interval; response was more rapid to the second vaccination, a high titer being reached in 7 days and maintained for another week before loss occurred. The rates of loss of antibody titer afterward were essentially identical in both instances.

The effects of one injection of vaccine adsorbed on various quantities of alum are shown in Fig. 2 (Experiment 2, Groups 4 and 5). The findings of Group 1 (Fig. 1, A) which received a single injection are repeated in Fig. 2 to compare more clearly the effects of 1.1% alum. It is seen again that the rate of response is relatively slow and, with all quantities of alum employed, the maximum titer was reached 2 weeks after vaccination. It is evident that the degree of response was considerably affected by the amount of alum employed. The highest mean titer observed with the vaccine in the presence of 0.3% alum was 277 and that with vaccine adsorbed on 1.1% alum was 147, a 2-fold difference in titer.

Discussion. Swine influenza virus vaccine adsorbed on alum elicited an antibody response in swine which differed in only two evident respects from the response to the identical kind and amount of the vaccine dispersed in saline solution. These differences were (1) a brief delay, approximately 1 week, in the

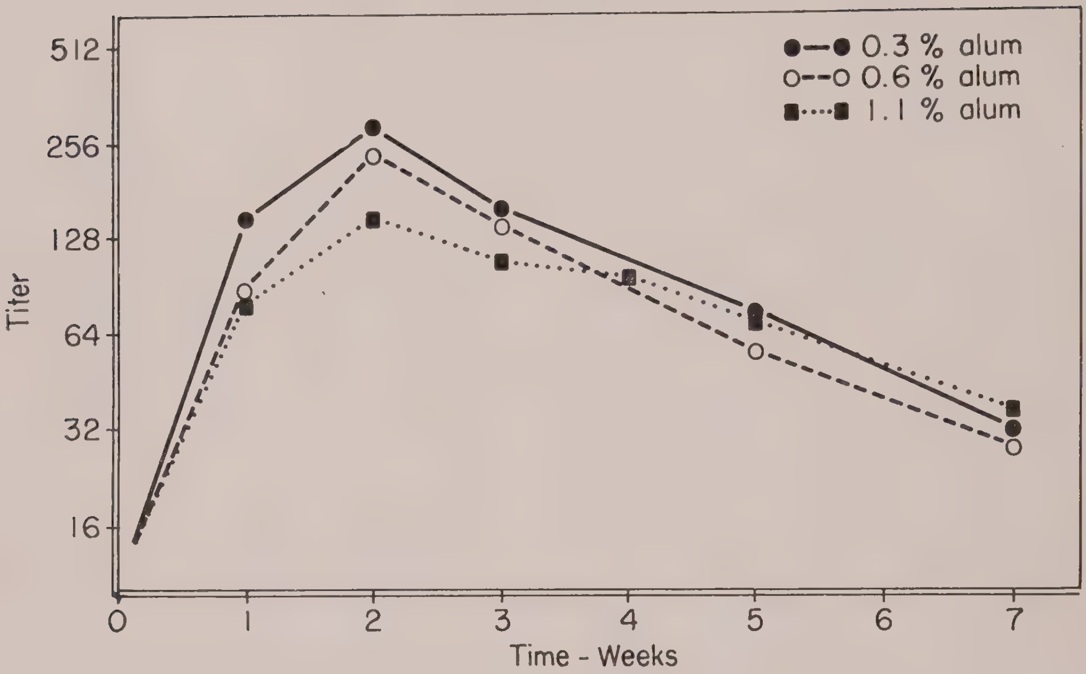

FIG. 2.
Effect of amount of alum present in vaccine on antibody response.

attainment of the maximum antibody level and (2) a lower antibody titer at the time of peak response. At no time was the response to the alum vaccine higher than that to the vaccine without alum. It is of interest to note that the maximum antibody titer induced by the vaccine with alum, which came at the interval of 2 weeks after vaccination, was essentially identical with the declining titer following the use of plain vaccine at the same interval. The results in swine were similar in this respect to those in man described by Bodily, Eaton and Corey,[10] for these authors measured antibody content at the interval of 2 weeks after vaccination with the two types of vaccine. Subsequent to the 2-weeks interval, the rates of antibody decline in swine were the same with the 2 vaccines and, consequently, the levels of antibody titer were alike with respect to time.

It was observed, further, that adsorption of the vaccine on alum may lessen the response to a given amount of the vaccine. In the present experiments, there appeared to be a definite relation between the height of response and the quantity of alum used. The response was least in the instance of 1.1% alum and greatest in that of 0.3% of the material. The present results as a whole indicate not only that adsorption of influenza virus vaccine on alum is of no benefit but that it may possibly be detrimental to the production and maintenance of anti-influenza antibody titer.

Summary. The antibody response of swine to formolized swine influenza virus adsorbed on alum is essentially the same as that to the vaccine dispersed in saline solution. The chief influence of the alum is a brief delay in the peak response. The higher concentrations of alum lessen the primary antibody response, but the subsequent antibody levels and the rates of diminution in titers are nearly identical with the two sorts of vaccine.

15190

Inhibition of *Salmonella* Cultures by Streptomycin.*

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Streptomycin is known to be active against various gram-negative bacteria, but little is known of its action on *Salmonella*. The present paper is a report of the *in vitro* inhibition of 412 *Salmonella* cultures by the agent. The cultures employed in the work included 154 distinct serological types which comprised all the presently recognized forms in the genus. Only one or two representatives of the majority of the types were studied since most of them occur very rarely. A number of the types which occur more frequently and which are better known were represented by a larger number of cultures.

The tests were performed by streaking tryptose agar plates which contained varying amounts of streptomycin with a 2 mm loopful of a saline suspension which contained approximately 100 million cells per ml. Streptomycin was added in amounts of 1, 2, 4, 8, 16, 32, and 64 units per ml of agar. Two samples of streptomycin which contained respectively 80 and 120 units per mg were used and similar results were obtained with both samples.† Streptomycin was added to the melted and cooled agar which was poured into plates and streaked immediately after hardening with freshly prepared bacterial suspensions. The plates were incubated 24 hours at 37°C. The smallest amount of streptomycin which suppressed all visible growth of the bacteria was recorded as the inhibitive concentration.

It was not found practical to space the dilutions of streptomycin more closely than the serial dilutions mentioned above since there was a gradual inhibition of growth as the amount of streptomycin increased. In some cultures a few resistant colonies developed on plates which contained the higher concentrations. In others complete inhibition was preceded by the occurrence of pin-point growth in one or two dilutions. Many of the tests were repeated several times and comparable results were obtained. The point of complete inhibition sometimes varied one dilution from test to test, *i.e.*, a given culture might be inhibited by 8 units in one test and by 16 units in a second. In most instances the end-point was the same in repeated trials.

Strain W of *E. coli* was used to control all tests and this culture was inhibited regularly by 4 units of streptomycin per ml of medium. The *Salmonella* strains in general were more resistant; the great majority of the types were inhibited by 8 to 16 units per ml. Space does not permit the separate listing of all the types tested but it may be said that with the exception of the types enumerated below and the cultures given in Table I, all the cultures were inhibited either by 8 or 16 units of streptomycin per ml. *S. typhi suis, S. duesseldorf, S. sendai* and *S. poona* were inhibited by 2 units of streptomycin per ml, while 4 units were required to inhibit *S. abortus-ovis, S. muenchen, S. dublin, S. rostock, S. moscow, S. blegdam, S. selandia, S. hvittingfoss, S. minnesota, S. meleagridis, S. orion,* and *S. pretoria.* Those cultures which were more resistant than the majority of the types required 32 units per ml to inhibit growth, Among this group were *S. amager, S. california, S. manhattan, S. altendorf, S. simsbury, S. mississippi, S. tennessee, S. florida, S. madelia,* and *S. heves.*

In Table I are listed 252 cultures of 19 of the types frequently encountered. Different cultures of the same type vary widely in their sensitivity to streptomycin and it is unwise to draw conclusions regarding the susceptibility of a given type unless several cultures are

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* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

† The samples of streptomycin were obtained through the courtesy of Merck and Company and their receipt is gratefully acknowledged.

TABLE I.
Inhibition of Salmonella Cultures by Streptomycin.

Type	No. of cultures tested	No. of cultures inhibited by units per ml of medium 1	2	4	8	16	32
S. paratyphi A	8		1	6	1		
S. paratyphi B	24			2	13	7	2
S. typhi-murium	28			2	13	9	4
S. derby	12		1	1	8	2	
S. paratyphi C	3				3		
S. choleræ-suis	12			5	5	1	1
S. choleræ-suis var. *kunzendorf*	16			1	7	8	
S. montevideo	12				4	7	1
S. oranienburg	13			2	9	2	
S. bareilly	11				6	4	1
S. newport	14		1	5	4	4	
S. typhi	8		1	6	1		
S. enteritidis	14		2	7	3	2	
S. pullorum	18	1	1	8	7	1	
S. gallinarum	16		1	5	7	3	
S. anatum	11				4	6	1
S. give	11				4	6	1
S. newington	11			1	2	4	4
S. senftenberg	10			1	4	4	1
E. coli W	1			1			

examined. Results obtained with only one or 2 cultures of a given type are only a tentative indication of the susceptibility of that type to the substance. In general, it may be said that *S. paratyphi* A and *S. typhi* were quite susceptible to the action of streptomycin while *S. pullorum* and *S. enteritidis* were inhibited by relatively low concentrations. The remainder of the commonly occurring Salmonella types were decidedly more resistant. It is of interest that not only was *S. enteritidis* one of the more easily inhibited types, but the closely related forms, *S. dublin, S. rostock, S. moscow,* and *S. blegdam,* were also inhibited by low concentrations of streptomycin.

Summary. The *in vitro* inhibition of 412 cultures of *Salmonella* by streptomycin was studied. All of the recognized types in the genus were included in the work. Of the commonly occurring types, *S. paratyphi* A and *S. typhi* were the most sensitive while *S. pullorum* and *S. enteritidis* were somewhat less susceptible. The remainder of the types which are found frequently were more resistant. The majority of the types required 2 to 4 times as much streptomycin to inhibit growth as did *E. coli* W. Different cultures of the same type often varied widely in their susceptibility to streptomycin.

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Cytotoxicity of Streptomycin and Streptothricin.

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The present study was done as part of a clinical and laboratory investigation of streptomycin, an antibiotic agent described by Schatz, Bugie, and Waksman.[1] Streptomycin has been found effective in the treatment of a number of experimental infections in laboratory animals.[2-10] In experiments reported by Feldman and Hinshaw[7] and by Feldman, Hinshaw, and Mann,[10] streptomycin was administered to guinea pigs over a long period without causing recognizable toxic reactions or tissue changes. Of several different lots of streptomycin used in the experiments of Feldman and Hinshaw, only one appeared to be toxic, indicating that the reactions observed were probably due to an impurity and not to streptomycin itself.

The tests of cytotoxicity reported in this paper were carried out on a number of samples of streptomycin before they were used for clinical investigation. In addition, a limited number of tests was also done with streptothricin, an agent described by Waksman[11] that is similar in many respects to streptomycin, especially with regard to its selective anti-bacterial activity. Robinson, Graessle, and Smith[5] have reported on the toxicity of streptothricin for mice. In another study they reported that some samples of streptomycin are several times less toxic than streptothricin in acute toxicity tests.[6] Feldman and Hinshaw[12] found streptothricin toxic for guinea pigs when it was given by repeated administration.

Experimental Methods. The tissue culture technic used has been described previously in detail.[13] Young adult male rabbits were used exclusively as a source of tissue, plasma, and serum used in the preparation of cultures. A serum-chick-embryo extract was made by extracting chick embryos of 8 days of incubation with rabbit's serum in the proportion of 1 embryo to 5 cc of serum. Fragments of rabbit's spleen measuring approximately 1 to 2 mm across were selected and were placed in Tyrode's solution in matched groups of 12 fragments each. In each experiment 1 group of 12 fragments was used in the preparation of the control series of cultures and each of the remaining groups comprised a test series. A solution of streptomycin or streptothricin* in Tyrode's solution was added to the tissue extract used in the preparation of the test cultures in an amount that would comprise 1/20

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[1] Schatz, Albert, Bugie, Elizabeth, and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

[2] Jones, Doris, Metzger, H. J., Schatz, Albert, and Waksman, S. A., *Science*, 1944, n.s. **100**, 103.

[3] Waksman, S. A., Bugie, Elizabeth, and Schatz, Albert, *Proc. Staff Meet., Mayo Clin.*, 1944, **19**, 537.

[4] Heilman, F. R., *Proc. Staff Meet., Mayo Clin.*, 1944, **19**, 553.

[5] Robinson, H. J., Graessle, O. E., and Smith, Dorothy G., *Science*, 1944, n.s. **99**, 540.

[6] Robinson, H. J., Smith, Dorothy G., and Graessle, O. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 226.

[7] Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clin.*, 1944, **19**, 593.

[8] Heilman, F. R., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 33.

[9] Heilman, F. R., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 169.

[10] Feldman, W. H., Hinshaw, H. C., and Mann, F. C., unpublished data.

[11] Waksman, S. A., *J. Bact.*, 1943, **46**, 299.

[12] Feldman, W. H., and Hinshaw, H. C., unpublished data.

[13] Herrell, W. E., Heilman, Dorothy, and Gage, R. P., *Am. J. M. Sc.*, 1943, **206**, 26.

* Samples of streptomycin and streptothricin were kindly furnished by Dr. Waksman and also by Dr. D. F. Robertson of Merck & Co., Inc. Additional samples of streptomycin were furnished through the kindness of Abbott Laboratories, Parke, Davis & Company, and the Upjohn Company.

TABLE I.

Effect of Different Preparations of Streptomycin on Migration of Macrophages and Growth of Fibroblasts in Cultures of Normal Rabbit's Spleen.

Streptomycin			Macrophages			Fibroblasts		
			Radius of migration, mean ocular micrometer units			Radius of growth, mean planimeter units		
Sample	Potency, units in 1 mg	Dilution × 1000	Control cultures*	Test cultures	% difference	Control cultures	Test cultures	% difference
1	200	1:1	269 ± 63	252 ± 42	— 6	755 ± 30	433 ± 21	—*43*†
		1:2	179 ± 9	192 ± 8	+ 7	1112 ± 63	968 ± 52	—13
		1:4	179 ± 9	174 ± 4	— 3	1112 ± 63	731 ± 66	—34
2	242	1:2	408 ± 21	373 ± 11	— 9	571 ± 31	433 ± 26	—*24*
		1:4	582 ± 20	567 ± 16	— 3	960 ± 27	863 ± 58	—10
3	333	1:2	408 ± 21	353 ± 9	—13	571 ± 31	396 ± 20	—*31*
		1:4	582 ± 20	550 ± 16	— 5	960 ± 27	810 ± 46	—*16*
4	270	1:2	408 ± 21	387 ± 18	— 5	571 ± 31	377 ± 29	—*34*
		1:4	414 ± 17	406 ± 14	— 2	751 ± 54	854 ± 50	+14
5	218	1:2	476 ± 20	382 ± 8	—*20*	927 ± 55	638 ± 30	—*31*
		1:4	414 ± 17	465 ± 20	+12	751 ± 54	784 ± 44	+ 4
6	102	1:2	582 ± 20	521 ± 36	—10	960 ± 27	789 ± 36	—*18*
		1:4	412 ± 17	384 ± 20	— 7	846 ± 30	827 ± 16	— 2
7	220	1:2	534 ± 24	517 ± 16	— 3	843 ± 37	655 ± 61	—*22*
		1:4	412 ± 17	402 ± 14	— 2	846 ± 30	847 ± 28	+ 0.1
8	150	1:2	534 ± 24	521 ± 21	— 2	843 ± 37	588 ± 47	—*30*
		1:4	412 ± 17	392 ± 14	— 5	846 ± 30	804 ± 32	— 5
9	369	1:1	467 ± 22	430 ± 14	— 8	936 ± 37	722 ± 39	—*23*
		1:2	467 ± 22	448 ± 17	— 4	936 ± 37	982 ± 61	+ 5
10	190	1:2	476 ± 20	417 ± 16	—12	927 ± 55	662 ± 15	—*29*
11	185	1:1	468 ± 17	506 ± 15	+ 8	805 ± 53	732 ± 19	— 9
		1:2	468 ± 17	480 ± 11	+ 3	805 ± 53	802 ± 27	+ 0.4
12	250	1:1	275 ± 11	237 ± 8	—*14*	852 ± 63	812 ± 27	— 5
		1:2	275 ± 11	265 ± 18	— 4	852 ± 63	809 ± 38	— 5
13	336	1:1	429 ± 7	364 ± 12	—*15*	730 ± 23	548 ± 28	—*25*
		1:2	429 ± 7	413 ± 10	— 4	730 ± 23	609 ± 30	—*17*
14	100	1:1		‡		730 ± 23	524 ± 23	—*28*
		1:2	429 ± 7	353 ± 12	—*18*	730 ± 23	691 ± 20	— 5

* The value following the ± is the standard error of the mean.

† Values in italics show significant variation from controls.

‡ Precipitate obscured cellular migration.

of the volume of the final tissue culture clot. A similar amount of Tyrode's solution was added to extract used in the preparation of control cultures.

Cultures were placed in D-5 Carrel flasks and consisted of 0.5 cc of heparinized rabbit's plasma, 1.0 cc of serum-chick-embryo extract and 4 explants of spleen which were placed in the medium before clotting occurred. Three flasks were prepared for each experimental condition. Preparations were incubated at 37°C for 5 days. Measurements of the migration of macrophages were made with an ocular micrometer at 96 hours of incubation by a method that has been described.[12] Fibroblastic growth was determined on the fifth day by projectoscopic methods. The average radius of the growth or migration zone in each group of test cultures was compared with that of the corresponding controls and the results were expressed in terms of percentage of inhibition or stimulation.

Results. Samples of streptomycin obtained from different sources were uniformly low in cytotoxicity (Table I). A concentration of 1:2000 of streptomycin caused a slight but significant inhibition of migration of macrophages in 2 instances and a decrease of fibroblastic growth in 9 of the 14 samples tested. One sample of streptomycin in a concentration of 1:4000 caused a slight but significant inhibition of the growth of fibroblasts but a similar concentration of the other preparations of streptomycin tested did not inhibit growth. Fibroblasts were more sensitive than macrophages to streptomycin except in samples 12 and 14. In the case of sample 14 the drug formed a precipitate in the culture medium. Particulate material frequently has a more toxic effect on macrophages than on fibroblasts because the particles are engulfed by the wandering cells.

The results of tests with 2 samples of streptothricin are recorded in Table II. Both samples were much more toxic for fibroblasts than for macrophages. Leukocytes that were present in the migration zone at 24 hours showed about the same sensitivity to streptothricin as did macrophages. The highest concentrations of the 2 samples of streptothricin that were not toxic for fibroblasts were 1:50,000 and 1:80,000 respectively.

TABLE II.
Effect of Different Concentrations of Streptothricin on Migration of Macrophages and Growth of Fibroblasts in Cultures of Normal Rabbit's Spleen.

Streptothricin		% variation from controls	
Sample	Dilution × 1000	Macrophages	Fibroblasts
1 325 units per mg	1:0.5	—100*	—100*
	1:1	— 57	—100*
	1:2	— 45	—100*
	1:10	— 4	—100*
	1:50	+ 3	+ 2
2 345 units per mg	1:20	+ 5	—100*
	1:40	+ 6	— 30
	1:60	+ 5	— 28
	1:80	+ 1	— 7

* 100 = complete inhibition of growth.

Comment. The low toxicity of streptomycin in tissue culture is in agreement with its relatively low toxicity for experimental animals.[2-4,7-10] Recently Reimann, Elias, and Price[14] have reported on the use of streptomycin in the treatment of typhoid fever. Serious toxic reactions due to streptomycin did not occur in their series of cases. Because all of the samples tested in the present study were relatively nontoxic, there was very little relation between the cytotoxicity observed and the potency of the preparation of streptomycin in units per milligram. It should be emphasized that the methods employed in the present investigation do not detect histamine activity, pyrogens or numerous other pharmacologic effects that do not interfere directly with the survival and growth of the cells being tested. The cytotoxicity of streptomycin is of about the same order as that of fairly pure penicillin tested by the same method.[15] The relatively low toxicity of streptothricin for leukocytes and macrophages and the fairly high toxicity for fibroblasts demonstrate the advantage of observing the effect of the substance being tested on more than one type of cell. The deleterious effect of streptothricin on fibroblasts is in agreement with the toxicity of streptothricin for guinea pigs observed by Feldman and Hinshaw.

Summary. Several different preparations of streptomycin were tested on cultures of rabbit's spleen and were found to have a uniformly low toxicity for wandering cells and fibroblasts. Streptothricin had a relatively low cytotoxicity for leukocytes and macrophages but showed a fairly high cytotoxicity for fibroblasts.

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[14] Reimann, H. A., Elias, W. F., and Price, A. H., *J. A. M. A.*, 1945, **128**, 175.

[15] Heilman, Dorothy H., unpublished data.

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Physiological Observations upon Larval *Eustrongylides.* IX. Influence of Oxygen Lack upon Survival and Glycogen Consumption.

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The larvæ of *Eustrongylides ignotus* are parasitic nematodes which lead in their natural habitat, encysted in *Fundulus heteroclitus,* a more or less completely aerobic life.[1,2] In view of the fact, however, that many parasitic round worms have extremely well developed anaerobic functions, it appeared of interest to investigate some reactions of this helminth to the lack of oxygen. The influence of the latter on the survival of the worms and their glycogen consumption will be discussed in the following sections. All experiments were conducted under strictly sterile conditions; the technic used in isolating worms under aseptic conditions has been described previously.[3]

Survival Under Lack of Oxygen. It has been shown[4] that larval *Eustrongylides* survive more than 30 months *in vitro* if kept at 20°C in rubber stoppered tubes containing sterile nutrient media. The two series upon which this statement was based have now come to an end and the results, not heretofore published, are summarized in Table I. That some worms survived as much as 4 years outside of their host is quite remarkable in view of the fact that most parasitic worms die rather rapidly when removed from their natural surroundings. Since the tubes containing these worms were tightly closed with rubber stoppers, it appeared likely that the gaseous atmosphere had undergone profound changes during the time of incubation. In the later stages of these series it became possible to analyze, in a van Slyke manometric apparatus, the gas present above the culture fluid after the worms had died. The average composition, in cases of larvæ having died after periods of 36 to 49 months after isolation, was 0.95% O_2 (extremes 0.37 and 1.73%) and 2.63% CO_2 (extremes 1.40 and 5.90%). These observations indicate that the worms tolerate a rather severe lack of oxygen.

TABLE I.
Survival of Larval *Eustrongylides ignotus* at 20°C in Sterile Media. Rubber-stoppered test tubes (25 x 150 mm) containing each 30 cc medium and one worm. Series A: Bacto-Broth 1.6%, NaCl 0.85%, glucose 0.5%. Series B: Bacto-Proteose-Peptone 1.0%, NaCl 0.85%, glucose 0.5%.

	No. of worms at beginning	No. of worms living after specified month 36	42	48	50
Series A	14	1	0		
,, B	12	5	3	2	0

The corresponding changes in atmosphere were more closely followed in another series in which the tubes were kept at 37°C. The same large size tubes were used as in the preceding series; the fluid medium consisted of 25 cc 1% peptone + 0.5% NaCl + 0.5% glucose and each tube contained only one worm. At the intervals specified in Fig. 1 4 worm tubes were used for analyses of the enclosed air. Despite the fact that the oxygen content diminished rather rapidly, falling below 1% after about 3 months, and that a pronounced accumulation of carbon dioxide occurred, all the worms were alive when the tubes were opened. It should be noted that the present data can only be used to show at what oxygen and carbon dioxide tensions the worms survive. They are insufficient to allow reliable calculations concerning the intensity of the gaseous exchange of the worms since control experiments showed that some changes in the atmosphere occur also in tubes containing no larva. These changes are probably due in part to the spontaneous oxidation of some constituent of the medium, in part to the oxidation of substances contained in the stopper.

While the worms are obviously not damaged

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[1] von Brand, T., *J. Parasitology,* 1938, **24**, 445.

[2] von Brand, T., *Biol. Bull.,* 1942, **82**, 1.

[3] von Brand, T., and Simpson, W. F., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 245.

[4] von Brand, T., and Simpson, W. F., *J. Parasitology,* 1944, **30**, 121.

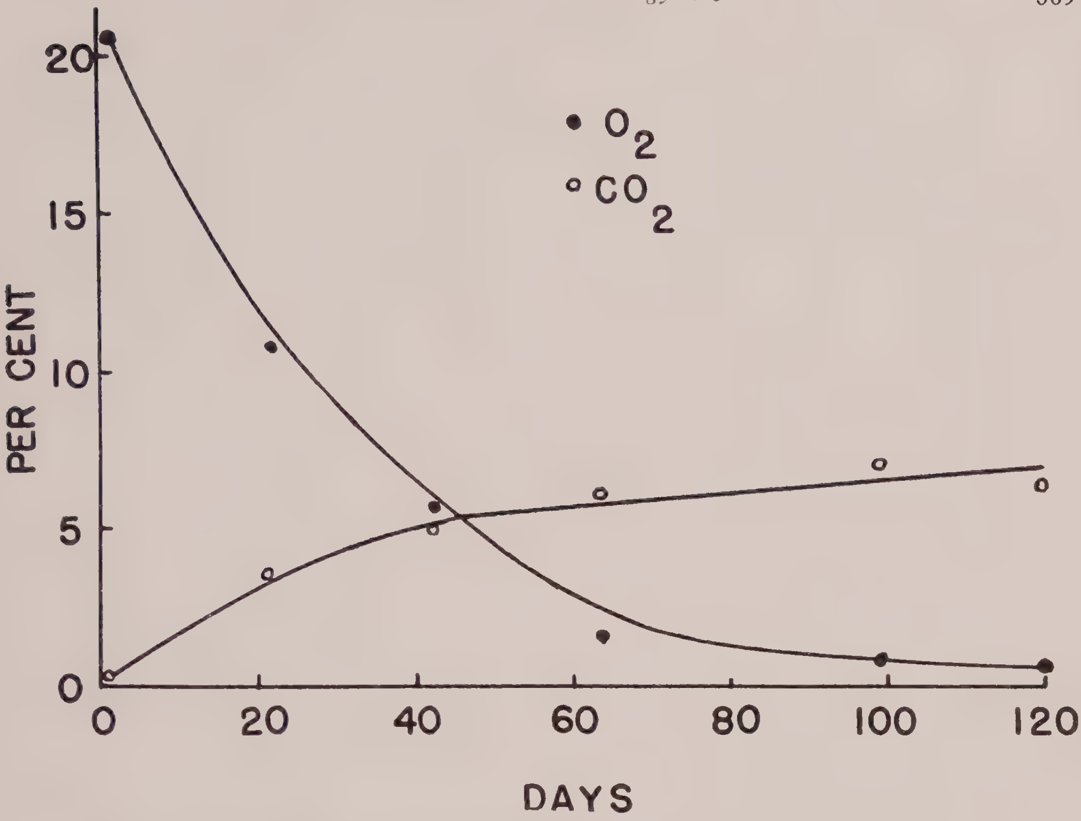

FIG. 1.

Oxygen and carbon dioxide in the atmosphere enclosed above the culture fluid in tubes containing larval *Eustrongylides* during incubation at 37°C.

by relatively low oxygen tensions, they do asphyxiate under complete lack of oxygen. A series of 30 was set up in standard size test tubes almost completely filled with Brewer's thioglycollate medium (Infusion from 37.5 g meat per 100 cc, 1% thio-peptone, 0.5% NaCl, 0.5% glucose, 0.2% dipotassium-phosphate, 0.1% Na-thioglycollate, 0.05% agar, 0.0002% methylene blue, initial pH 7.5) which were tightly closed with rubber stoppers. That anaerobic conditions actually prevailed is indicated by the fact that the methylene blue remained completely reduced throughout the course of the experiment. The average survival of these worms was 18 days (extremes 4 and 25 days). It now became necessary to ascertain whether the brief survival was actually due to the lack of oxygen, or to a toxic action of some constituent of the medium. To this purpose a new series was established in test tubes filled only to about one-third with the above medium and these tubes were closed with cotton stoppers only. In all 21 tubes employed the movements of the individual worm was sufficient to mix the contents to such an extent that enough oxygen diffused from the surface to oxidize the methylene blue throughout the entire column within 2 days. The average survival of these larvæ was 98 days (extremes 10 and 161 days). The difference between the 2 series proves that the ingredients of the medium are non-toxic and not responsible for the early death of the anaerobically kept worms.

Influence of Anaerobiosis on Glycogen Consumption. In order to study the influence of lack of oxygen on the glycogen consumption of the larvæ, glycogen determinations according to von Brand's[5] modification of Pflueger's method were carried out on freshly isolated worms and on worms kept for 6 days at 37°C in various solutions under aerobic and anaerobic conditions respectively (Table II). The test tubes were not closed by a stopper or cotton plug, but their sterility was maintained by placing over their open end an inverted loosely fitting glass vial, thus allowing an easy interchange with the surrounding atmosphere. Anaerobic conditions were established by placing the rack supporting the tubes into a desiccator in which the oxygen was absorbed by an alkaline solution of pyrogallol (Buchner's procedure). The completeness of the absorption was ascertained by withdrawing a gas sample and analyzing it in a van Slyke manometric apparatus.

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[5] von Brand, T., *Skandin. Arch. Physiol.*, 1936, **75**, 195.

The results summarized in Table II show that, under aerobic conditions, the glycogen consumption was greatest in pure saline, indicating that the nutritive material contained in the other series exerted a glycogen sparing action. This confirms earlier observations extending over longer periods.[4] Under anaerobic conditions, on the other hand, no such difference could be observed. The worms are apparently unable to utilize either peptone or sugar contained in the medium when oxygen is completely lacking. The evidence for this assumption is perhaps not quite convincing; direct determinations in the medium were not carried out, because the evaporation taking place under the condition of the experiments excludes the possibility of gathering reliable data. The glycogen consumption itself under anaerobic conditions was about three times that found under aerobic conditions, if for such comparison only the glycogen disappearance in a non-nutritive medium is used. This, of course, is due to the fact that an anaerobic carbohydrate breakdown yields but little energy.

The pH of all the solutions employed decreased during the experiments and this decrease was much more pronounced under anaerobic than aerobic conditions. This is in agreement with the previously reported excretion of acids by anaerobically kept Eustrongylides larvæ.[1]

A number of comparable series were carried out at 20°C. Despite the fact that they extended over 3 weeks each no definite conclusions could be reached. At this temperature

TABLE II.

Glycogen Consumption of Larval *Eustrongylides ignotus* Under Aerobic and Anaerobic Conditions, 37°C.

Medium	Beginning					After 6 days aerobiosis							After 6 days anaerobiosis						
	No. of det.	No. of worms	Avg wt per worm, mg	Glycogen %	pH of medium	No. of det.	No. of worms	Avg wt per worm, mg	Glycogen %	Glycogen change mg/g worm/day	pH of medium	pH change	No. of det.	No. of worms	Avg wt per worm, mg	Glycogen %	Glycogen change mg/g worm/day	pH of medium	pH change
NaCl 0.85%	4	20	65	8.28	6.7	4	21	69	6.8	—2.4	5.6	—1.1	4	24	56	3.9	—7.3	4.3	—2.4
NaCl 0.85% + 0.2% glucose	4	23	90	7.81	6.5	4	24	57	7.1	—1.1	5.31	—1.2	4	24	57	4.0	—6.4	4.41	—2.1
Peptone 0.5% + 0.5% NaCl	4	24	56	6.61	7.0	4	20	69	6.6	+0.01	6.02	—1.0	4	24	62	2.5	—6.9	5.01	—2.0
Peptone 0.5% + 0.5% NaCl + 0.2% glucose	4	24	58	7.11	7.0	4	26	66	6.8	—0.5	5.96	—1.1	4	26	60	2.7	—7.3	5.1	—1.9

the daily rate of aerobic glycogen consumption is very low[4] and consequently the variations in glycogen content between various batches of worms may falsify any result. It was unfortunately not possible to conduct the experiments over a longer period, because too many worms die after 3 weeks anaerobiosis.

Summary. Some specimens of larval *Eustrongylides ignotus* could be maintained for 4 years *in vitro*. The worms survived for long periods in media showing a pronounced partial lack of oxygen, but they did not tolerate complete absence of oxygen for very long periods. Anaerobically kept worms seemed not to feed but they consumed from their body reserve about three times as much glycogen as worms starving under aerobic conditions.

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Effect of Acute A-V Fistula on Circulation Time and Auricular Pressure in Dogs.

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The physiological effects of A-V fistula have long been the subject of extensive experimental investigation. Added impetus has been given to this work by successive wars. The existence of such effects as the Nicoladoni-Branham phenomenon, increased pulse pressure, decreased diastolic pressure, and the relationship of these changes to the size and location of the fistula, have been definitely established. On the other hand, another group of physiological effects or the explanation thereof, such as increased cardiac output,[1,2,3] increased blood volume, and increased pressure in the large veins proximal to the fistula,[4,5] have been matters of considerable controversy. Finally there are some effects which have been studied incompletely or not at all. The present investigation was undertaken to determine the effect of A-V fistula, first, on femoral and jugular circulation times and the relationship between the two, and second, on auricular pressures.

Method. Thirty observations were made on 5 dogs. The animals were anesthetized with nembutal intravenously, 1 grain per 5 lb of body weight. The right femoral artery and vein, and right jugular vein were exposed under rigid aseptic conditions. Right femoral arterio-venous fistulæ were made in the manner described by Holman.[4] Femoral and jugular circulation times, using the cyanide method described by Robb and Weiss,[6] were determined before and after the fistula was made. All injections into the femoral vein were made at a point just proximal to the site of the fistulous opening into the vein. The injections into the jugular vein were made at a point midway between the lower edge of thyroid cartilage and sternum. Next, auricular pressures were determined with the fistula open and closed. A No. 10 French catheter, with tip cut off, was introduced into the right auricle by way of the right jugular vein according to the method of Richards *et al.*[7] Heparin was added to the saline in the catheter to prevent clotting. In one dog (No. 5) auricular pressures were not studied until 5 months after the fistula was made. All readings were made in duplicate and mean readings were determined.

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[1] Lewis, T., and Drury, A. N., *Heart*, 1923, **10**, 301.

[2] Ellis and Weiss, S., *Am. Heart J.*, 1929-1930, **5**.

[3] Grollman, A., *Cardiac Output*, Hopkins, 1932.

[4] Holman, E., *Arterio-venous Aneurysm*, Macmillan Company, 1937.

[5] Reid, M. R., and McGuire, J., *Annals Surg.*, 1938, **108**, 643.

[6] Robb and Weiss, S., *Am. Heart J.*, 1932-1933, **8**.

[7] Richards, Cournand, Darling, Gillespie, and Baldwin, *Am. J. Physiol.*, 1942, **136**, 115.

TABLE I.

	Femoral circulation times					Jugular circulation times				
	Before fistula		After fistula		Diff. + or —	Before fistula		After fistula		Diff. + or —
Dog No.	Readings	Mean	Readings	Mean		Readings	Mean	Readings	Mean	
5	9.8 9.0	9.4	6.5 6.9	6.7	—2.7	6.2 6.2	6.2	8.8 8.0 8.7	8.5	+2.3
17	8.0 7.8 8.0	7.9	6.0 6.8 6.6	6.5	—1.4	6.4 6.5	6.5	7.5 7.5	7.5	+1.0
18	8.4 8.0	8.2	5.6 5.6	5.6	—2.7	7.3 7.7 7.0	7.3	7.2 7.0	7.1	— .2
19	7.5 7.5	7.5	5.2 4.5 4.5	4.7	—2.8	6.4 5.8 5.8	6.0	5.1 5.3	5.2	— .8
20	9.8 10.0	9.9	7.0 6.9	7.0	—2.9	9.8 8.2	8.0	7.9 8.1	8.0	±0
Mean	8.52		6.0			6.9		7.3		
Standard deviation	±0.826		±0.777			±1.267		±1.338		
"t"	6.3					0.81				
P.	<0.01					>0.4				
	Significant difference					No significant difference.				

Results. Studies of the femoral circulation times showed that a statistically significant speed-up of circulation occurred when a femoral arterio-venous fistula was produced in the vascular tree. The mean reduction in femoral circulation time in the 5 dogs was 2.64 seconds or 30.9%. On the other hand, jugular circulation times gave varying results. A-V fistula in 2 dogs caused a mean increase of the jugular circulation time amounting to 1.63 seconds. In 2 dogs it caused an insignificant decrease of the jugular circulation time, and in one dog no change was observed. (Table I.)

In the 5 dogs there was a rise in auricular pressure when the fistula was open. The mean increase in pressure amounted to 3.2 cm of H_2O, or a rise of 71.1%. (Table II.)

TABLE II.
Mean Auricular Pressure—CMs H_2O in Experimental A-V Fistulæ.

Dog No.	Fistula closed	Fistula open	Difference
17	4.7	8.8	+4.1
18	3.5	6.4	+2.9
19	6.2	9.3	+3.1
20	3.7	6.4	+2.7
Mean	4.5	7.7	
Standard deviation	±1.06	±1.33	
"t."	3.2		
P.	<0.05		
∴ Difference is significant.			

The measurements of auricular pressures obtained in Dog No. 5 were not included, because no control readings were obtained when the fistula was first made. The rise in pressures in the 4 dogs with acute fistulæ was statistically significant.

Discussion. The results indicate that the introduction of a femoral A-V fistula into the circulation will cause the following two important changes: first, speed-up of circulation in the fistula circuit, and second, an increase in auricular pressure. These two observations together suggest that a greater volume of blood is being brought to the right auricle during a given period of time. Therefore, if the heart is not failing, it must put out more blood in the same given period of time. This provides indirect evidence that an A-V fistula causes an increase in cardiac output.

On the other hand, statistical analysis demonstrated that the circulation time in the jugular circuit showed no significant change. It is known that the jugular circulation time is practically a measurement of the rate of flow through the pulmonary circulation to the carotid sinus. Since the jugular circulation time did not change significantly, it is obvious that the increase in rate of blood flow occurred primarily in the segment of the fistula circuit extending from the fistula to the heart.

The significant increase in auricular pressure caused by an A-V fistula seems to substantiate the work of Holman, who maintained that there was an increase of pressure in the proximal venous portion of a fistula circuit.

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Value of Sunflower Seed Protein.

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From statements of a recent report, the nutritive value of sunflower seed meal would appear to depend more on its content of B-complex vitamins than of protein, even though 53% of the product studied was protein.[1] In a study of the biological value of the protein of sunflower seed meal by the nitrogen balance method, it was concluded that this protein is inferior to that of soybean meal, and is "in the same class as the better cereals oats, wheat, and barley."[2] These reports indicate that sunflower seed meal is of only mediocre value as a source of protein. On the other hand, the amino acid analysis of sunflower seed meal compares very favorably with analyses of beef muscle and other tissue proteins of high value.[3,4] On the basis of known chick requirements,[5] the meal should be adequate as a sole source of protein with the possible exception of a slight deficiency of lysine. The obvious disagreement between the biological and analytical data led us to conduct further tests with the chick.

Two-week-old White Leghorn chicks were segregated into groups on the bases of weight and gain during the second week after hatching, and were given the experimental diets, which were (except for the protein sources) identical with those previously used.[6,7,8] All the protein (20% of the diet) was supplied by a South American sample of sunflower seed meal which contained 46.7% protein (N × 6.25) or by a laboratory-prepared meal which contained 39.9% protein. Supplemented casein and deboned sardine meal served as positive controls in these experiments.

The data on sunflower seed meal presented here (Table I) indicate quite clearly that the value of this product for chick growth and efficiency of feed utilization is very high. It compares favorably with other recognized complete sources of amino acids for the chick. In fact, the protein of sunflower seed meal is the most complete vegetable protein for chick growth that we have studied. This result is in agreement with the amino acid analyses of the protein[3,4] and the known chick requirements.[5] It is apparent that even lysine is present in adequate amount, although lysine is deficient in many cereal and oilseed meal proteins.

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[1] Day, A. G., and Levin, E., *Science*, 1945, **101**, 438.

[2] Mitchell, H. H., Hamilton, T. S., and Beadles, J. R., *J. Nutrit.*, 1945, **29**, 13.

[3] Block, R. J., and Bolling, D., *Arch. Biochem.*, 1945, **6**, 227. Also earlier private communications from R. J. Block.

[4] Grau, C. R., and Almquist, H. J., *Arch. Biochem.*, 1945, **6**, 287.

[5] Almquist, H. J., *Trans. Am. Assn. Cereal Chemists*, 1945, **3**, 158.

[6] Grau, C. R., and Almquist, H. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 187.

[7] Almquist, H. J., and Grau, C. R., *Poultry Sci.*, 1944, **23**, 342.

[8] Grau, C. R., and Almquist, H. J., *Poultry Sci.*, 1944, **23**, 486.

TABLE I.
Comparative Gains on Chicks on Diets Containing the Equivalent of 20% Crude Protein Exclusively from Sunflower Seed Meal, Sardine Meal, or Casein Plus Certain Amino Acids.

Exp. No.	Amino acid source	Chicks per group, No.	Duration of exp., days	Avg total gain, g	Rate of gain per day, %	Gain per g feed consumed, g
1	Sunflower seed meal, com'l.	5	11	62	5.3	
	Sardine meal*			67	5.7	
2	Same	6	12	121	5.7	0.36
				112	5.4	0.34
3	Sunflower seed meal, com'l.	10	21	147	4.8	0.38
	Casein, arginine, cystine, glycine			147	4.8	0.39
4	Sunflower seed meal†	10	9	76	6.5	0.42
	Sunflower seed meal,† lysine			80	6.5	0.43
	Casein, arginine, cystine, glycine			57	5.5	0.43

*This product was deboned by floating on carbon tetrachloride; it contained 85.0% protein (N × 6.25).
†Sample prepared in the laboratory from sunflower seed.

The amino acids which are required by the chick for optimal growth are greater in number and equal to or greater in quantity than those required by the rat.[5] The disagreement between the rat biological data and the amino acid and chick biological data may be due to the use of poor quality samples in the rat tests.

Summary. The protein of sunflower seed is a complete single source of amino acids for the growth of the young chick, when fed to provide 20% protein in the diet.

15195

"Thermostable" Thromboplastin from Human Placenta and Chicken Brain.*

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In the course of a study of the hormones of placenta we observed that extracts of this organ exhibit a strong blood-coagulating action.[1] The active substance resembled thromboplastin, the factor which by converting prothrombin into thrombin activates the reaction fibrinogen → fibrin and thus promotes the coagulation of blood. It could be shown that this active agent is relatively thermostable.

Heat stable thromboplastin from placenta is of special therapeutic interest, since its use makes it possible to avoid the side-effects which attend the local or parenteral administration of thromboplastin from heterogenic source.[2,3] Since thromboplastin of birds is less active on mammal blood than is mammal thromboplastin and vice versa, it seems probable that the two are different. A comparison between these two thromboplastins was therefore carried out.

The properties of thromboplastin have been studied in the last few years by various laboratories. A very extensive research has been

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* Generously aided by a grant from the Rockefeller Foundation.

[1] Finkelstein, M., *Nature*, 1945, **155**, 202.

[2] Eley, R. C., Green, A. A., and McKhann, C. F., *J. Pediat.*, 1936, **8**, 135.

[3] Edsall, Y. T., Ferry, R. M., and Armstrong, S. H., *J. Clin. Invest.*, 1944, **23**, 557.

undertaken by Chargaff *et al.*[4–8] These authors isolated substances of a thromboplastic nature from animal tissues by a variety of methods. Using water as extracting medium and purification of the extract by differential centrifugation procedures they obtained thromboplastic preparations described as "high molecular lipoprotein." By means of organic solvents thromboplastic preparations belonging to the phosphatide group were separated. Both preparations proved relatively thermostable.[5,6]

Experimental. Preparation of Thromboplastin from Placenta. Placenta was perfused with tap-water and then minced with sand. 20 g of the mince was extracted with 20 cc cold distilled water. The suspension was centrifuged. The supernatant fluid was sucked through a Seitz filter, and the filter pad was then washed with distilled water until the wash-water became colorless. The filter pad was broken up under cold distilled water and the suspension was filtered through filter paper. The resulting almost colorless, opalescent filtrate contained the thromboplastin used in the present study. It contained 1.4 mg dry matter per cc and exhibited a protein reaction with sulfo-salicylic acid.

Preparation of Thromboplastin from Chicken Brain. Twenty g of chicken brain was freed from grossly visible blood vessels and minced in a mortar with sand in 20 cc of water. The mash was pressed through gauze. After centrifugation the supernatant was diluted with equal parts of distilled cold water and was centrifuged again. The upper layer was removed with a pipette and filtered through a Seitz filter. Subsequent steps were as described for the preparation of thromboplastin from placenta. The resulting solution contained 1 mg dry matter per cc.

The thromboplastic activity of the preparation from placenta was tested on oxalated human plasma, and the preparation from chicken brain was tested on native[†] chicken or rooster plasma. (The thromboplastin from chicken brain shows very little activity when tested on human oxalated plasma, and high activity when tested on chicken plasma; thromboplastin from human sources exhibits the reverse behavior.)

The "coagulation time" of oxalated human plasma was tested by the addition of 0.1 cc thromboplastin to 0.1 cc plasma. After 5 minutes of incubation at 37°C 0.1 cc 0.02 M calcium chloride was added and the tube was shaken vigorously. The time of clot formation was recorded with a stop watch.

For the determination of the "coagulation time" of chicken plasma 0.1 cc of chicken plasma was transferred to a small clean tube kept in a water bath at 37°C. After 5 minutes of incubation 0.05 cc of thromboplastin was added with a micropipette and the tube was shaken vigorously. The time from the addition of the thromboplastin until clot formation was noted with a stop watch. Every experiment was performed at least 3 times. Only plasma specimens whose coagulation times without thromboplastin were not less than 2 hours were used.

Results. As we have reported in a preliminary paper[1] the purified aqueous placenta extract with strong thromboplastic activity is not markedly affected by heating for 5 minutes in a boiling water bath. This holds true, however, only in tests carried out with fresh oxalated human plasma. When the experiment is performed with plasma stored for some days in a refrigerator, the coagulation time in the presence of heated thromboplastin is greately increased in comparison to that found with an unheated preparation (Table I).

The thromboplastic preparation from chicken brain is thermostable in the sense that it withstands heating in a boiling water bath for 5 minutes, but it differs from thromboplastin from placenta in some other respects. Preparations of thromboplastin from placenta

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[4] Chargaff, E., Moore, G. H., and Bendich, A., *J. Biol. Chem.*, 1942, **145**, 593.

[5] Chargaff, E., *J. Biol. Chem.*, 1944, **155**, 387.

[6] Chargaff, E., Bendich, A., and Cohen, S., *J. Biol. Chem.*, 1944, **156**, 161.

[7] Cohen, S., and Chargaff, E., *J. Biol. Chem.*, 1940, **136**, 243.

[8] Cohen, S., and Chargaff, E., *J. Biol. Chem.*, 1941, **140**, 689.

[†] Chicken or rooster plasma may be kept liquid without addition of a decalcifying agent if drawn carefully and stored in a clean container in a refrigerator.

TABLE I.
Effect of Heated and Unheated Thromboplastin on Coagulation Time of Fresh and Stored Plasma.

Age of plasma in days	Coagulation time in sec.	
	Unheated thromboplastin	Heated thromboplastin
0	12	12
0	13	13
0	15	18
2	18	23
3	21	28
7	42	185
28	70	> 10 min.

were affected very slightly or not at all by heating. The activity of the heated preparation remained constant when the preparation was kept in a refrigerator. The chicken brain preparations, on the other hand, were affected much more strongly by heating. The coagulation time of heated brain preparation was in some cases about twice that of the unboiled preparation. If, however, a heated preparation was kept in the cold for some days and then tested, its original thromboplastic power was found to be completely restored (Table II).

TABLE II.
Reactivation of Heated Thromboplastin from Chicken Brain Kept at 4-6°C.

Time between heating of thromboplastin and test performed	Coagulation time in sec.	
	Unheated thromboplastin	Heated thromboplastin
Immediately	28	52
1 day	28	30
7 days	26	28

Enzyme chemistry knows similar cases. If, for example, trypsin is inactivated by heating for a short time, reactivation of the enzyme on cooling is complete.[9]

Native chicken or rooster plasma shows no prolongation of coagulation time after storage in a refrigerator for a considerable period of time (1 month) when tested with boiled and reactivated or unboiled chicken brain thromboplastin (Table III). On the other hand, oxalated human plasma, as stated previously, shows when stored a prolongation of its coagulation time as determined on a boiled preparation of thromboplastin from placenta.

Discussion. This paper shows that the "coagulation time" of stored oxalated plasma is greater in the presence of heated thromboplastin than in the presence of unheated thromboplastin. On the other hand, the "coagulation time" of fresh oxalated recalcified plasma or stored "native plasma" (chicken plasma) is the same for heated and unheated thromboplastin.

TABLE III.
Effect of Heated and Unheated Thromboplastin from Chicken Brain on Stored Chicken Plasma.

Age of plasma, days	Coagulation time in sec.	
	Unheated thromboplastin	Heated thromboplastin
0	30	31
1	29	31
3	31	30
30	29	32

An explanation of this phenomenon is suggested by the findings of Quick.[10] Quick believes that prothrombin is composed of two components A and B, which are bound by calcium and thus protected. In decalcified (oxalated) plasma the component A undergoes destruction and gradually disappears from the plasma. "Native" plasma, however does not change in its prothrombin content on storage as it is protected by calcium.

In view of these and our own findings, we assume that thromboplastin too is composed of two components, I and II. One of these is thermostable (I) and withstands heating for 5 minutes, while the other (II) undergoes destruction. The thermostable component (I) acts on plasma in which the prothrombin complex (parts A and B) is still intact, *e.g.*, on fresh oxalated plasma or on "native" plasma (chicken plasma), but does not act on plasma in which the component A of prothrombin is destroyed (stored oxalated plasma). The two components (I and II) of thromboplastin together, *i.e.*, in an unheated thromboplastic preparation, exhibit activity towards stored oxalated plasma. In other words, they activate the component B of prothrombin.

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[9] Sizer, I. W., *Advances in Enzymology*, 1943, Vol. III, 35-60.

[10] Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

It may be concluded, therefore, that the thermostable part of thromboplastin (I) is responsible for the activation of component A of prothrombin. The component B may be activated either by the two parts of thromboplastin together, or by the thermolabile one alone, but the mechanism of activation of component B has not yet been ascertained.

The observation of certain workers that thromboplastin is definitely destroyed by heat may be explained by the fact that the behavior towards heat depends on the method of the preparation. It seems possible that inactivation is due to presence together with the thromboplastic factor of proteins which by denaturation eliminate the active agent.

Since our thromboplastin preparation is thermostable in respect of its ability to coagulate fresh plasma or blood, it is particularly well suited for diagnostic and therapeutic use. It has been used by us for the following purposes: (1) To determine prothrombin time. The aqueous solutions are set up in ampoules for use as necessary; (2) To obtain local stoppage of blood flow; (3) To fix skin transplants. The titrated plasma is placed over the wound together with thromboplastin and calcium and the clot which forms within a few seconds serves as an adhesive for the skin graft (Sano[11]). The advantages of our preparation are its availability in an ampoule, and the fact that it contains human protein. (The undesirable possible by-effects of the use of non-human protein are thus eliminated.) Our clinical experience of this technic will be discussed jointly with Prof. Mandl elsewhere. (4) For injection into man. Since the preparation contains homologous protein undesirable reactions are avoided. Experiments on this aspect are under way.

A sterile and thermostable thromboplastin from chicken tissue may prove useful in plasma culture. The preparation from chicken brain elicits coagulation of chicken plasma within a few seconds. The thromboplastin can be heated and in this way accompanying growth-promoting substances can be destroyed. The coagulation of plasma in tissue cultures is usually induced by the addition of fresh chicken embryo extract which may contain growth-promoting substances to the culture medium. Research on the effect of growth-promoting substances in tissue cultures has been complicated by this circumstance.

Summary. A method of preparation of thermostable thromboplastin from human placenta and of thermolabile but reactivable thromboplastin from chicken brain is described. The properties and differences of these preparations are discussed. Human placenta thromboplastin is not inactivated by heating at 98°C for 5 minutes. Chicken brain thromboplastin is inactivated by the heat treatment but is subsequently reactivated when it is stored at 4-6°C. The different activities of heated and unheated placenta and chicken brain thromboplastin on stored oxalated human plasma and native chicken plasma are explained by the assumption that thromboplastin is composed of two factors, one of them thermostable and the second thermolabile. Clinical, surgical, and research applications of thermostable preparations of thromboplastin are discussed.

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[11] Sano, M. E., *Am. J. Surg.*, 1943, **61**, 105.

15196

An Improved Cage for Nitrogen Studies with Mice.

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During a study of nitrogen balance in mice on different diets, it was found that the experimental arrangements described in the literature for similar experiments were not entirely satisfactory. In such studies the total nitrogen ingested and excreted must be determined. Spillage of food, fouling of the food in the container, loss of nitrogen from excreta via ammonia and poor ventilation are some of the factors contributing to error in such determinations.

The cage described by Bittner[1] is not suitable for this investigation, particularly with the diets used in our experiments.

In our first experiments, beaker cages similar in principle to the glass containers described by White[2] were used. The feeding dishes were equipped with screw lids having a small orifice and were held in position by metal collars attached to the floor. In spite

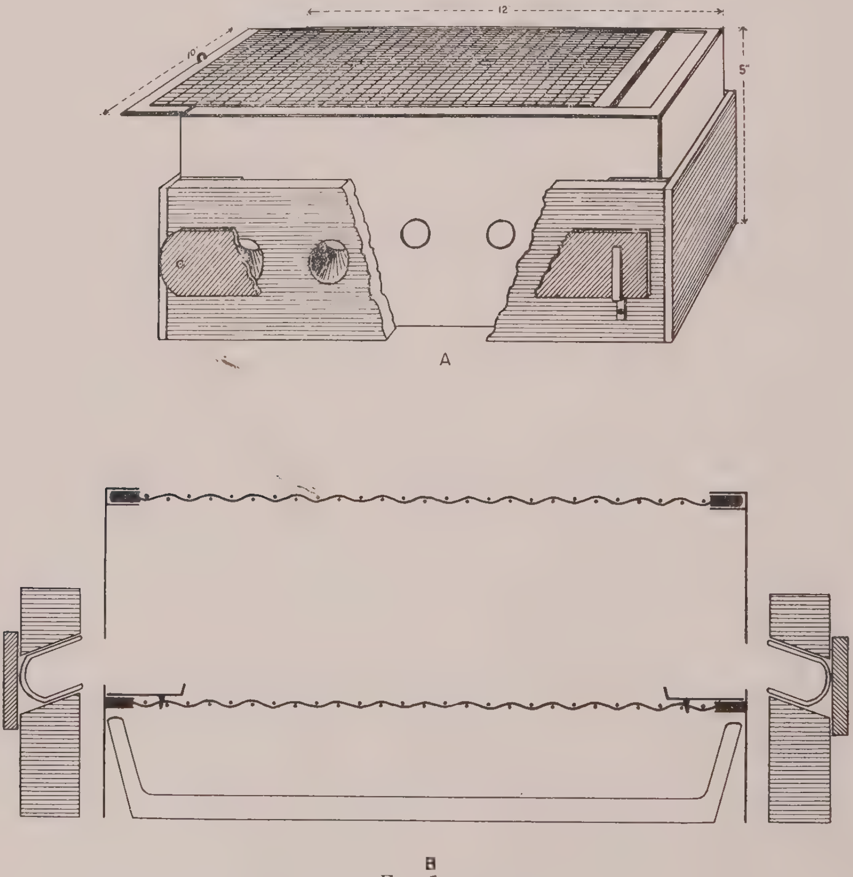

Fig. 1.
A. Exterior aspect of feeding cage.
B. Cross-section showing glass collecting tray.

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[1] Bittner, J. J., Chapter 13, *Biology of the Laboratory Mouse*, G. O. Snell, editor, Blakiston Company.

[2] White, F. R., *Cancer Research*, 1945, **5**, 265.

of these precautions food spillage invariably occurred. Where nitrogen intake is determined from the weight of food eaten or where the caloric intake is being followed, spillage may be a large source of error. It was found that the individual cages used by White or by ourselves gave incomplete separation of the urine and feces. Difficulty was also experienced in cleaning the fine screens. Poor ventilation is evident when the feces are not removed daily. Loss in ammonia, however, was reduced by putting 25% sulphuric acid in the bottom of the mouse jar. It was observed that mice kept in individual cages ate less and did not maintain their weight as well as mice kept together in larger cages. Similar observations have been made by Brunschwig *et al.*[3]

To overcome the objections inherent in these methods of management of the mice, the metabolism chamber shown in Fig. 1 was devised. It was designed to accommodate 10 mice. It consists of a metal cage 12″ x 10″ x 5″ with a single floor of No. 3 gauge screen, 2 inches from the bottom. This fits over a glass tray. Five feeding holes, ⅝″ in diameter, are in each of 2 opposite walls. These holes are opposite recesses in a 1-inch board which forms part of an outer frame into which the metal cage fits, but which is offset ⅜″ from the metal wall. The recesses, ⅞″ in diameter, are depressed at an angle of 20° and are receptacles for the glass food cups. A wooden gate acts as the back-stop and enables the food cups to be inserted one at a time. The glass food cups are cut to fit the oblique hole so that there is a space of 3/16″ between the edge of the cup and the wall. Inside and touching the wall below each set of feeding holes is a movable metal tray kept in a position by 2 metal projections into the screen floor. A sliding screen cover furnishes adequate ventilation. Water bottles are inserted through the screen mesh. The whole assembly sits on a sheet of clean paper.

This experimental set-up solves the problem of spillage and fouling of the food. The mouse can reach the bottom of the feed cup but does not pull food into the cage. Any food pulled out of the cup drops between the metal wall and the wooden frame to the clean paper. Occasionally some food may stick to the head of the animal and subsequently be dislodged when the mouse again pokes its head into the feed hole. This material drops to the metal tray, and is recovered. Spillage was reduced to a minimum when the diet had the consistence of toothpaste.

Summary. A cage for the proper management of mice during nitrogen balance studies is described. The arrangement eliminates spillage of food, fouling by excreta, loss of ammonia, and other sources of error.

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[3] Brunschwig, A., and Rasmassen, R. A., *Cancer Research*, 1941, **1**, 371.

15197 P

Autoantibodies in Rheumatic Fever.*

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As reported previously[1,2,3] autoantibodies to kidney can be produced by immunization of animals with mixtures of group A streptococci and kidney of the same species. The streptococci thereby confer antigenicity on renal material which, as such, is nonantigenic in the homologous species. The autoantibodies to kidney thus formed react specifically with plain normal homologous kidney as demonstrated *in vitro* by the collodion par-

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* This investigation was aided by grants from the Commonwealth Fund.

[1] Cavelti, Philip A., and Cavelti, Else S., *Arch. Path.*, 1945, **39**, 148.

[2] *Idem., A~ch. Path.*, 1945, **40**, 158.

[3] *Idem., Arch. Path.*, 1945, **40**, 163.

ticle technic. Their *in vivo* effect is the production of glomerulonephritis by means of their reaction with the immunized animal's own kidney.

It is thus conceivable that human glomerulonephritis also might be due to the pathogenic action of specific nephrotoxic autoantibodies produced as a response to an antigen resulting from a combination of streptococcal and renal material during the streptococcal infection, which commonly precedes glomerulonephritis by about 2 weeks.

Investigations as to whether substances from other homologous tissues, besides kidney, also might be rendered antigenic by combination with streptococci, and as to the lesions resulting from such autoantibodies, are under way and will be reported later; however, it can be stated that autoantibodies to heart, skeletal muscle, and connective tissue can be produced in this manner.

These results together with the fact that, similar to glomerulonephritis, rheumatic fever also is preceded by a streptococcal infection, suggest the possibility that autoantibodies to certain tissues might play a role in this disease.

Attempts were undertaken to demonstrate in the serum of patients with acute rheumatic fever, autoantibodies to human heart, the organ most regularly affected by this disease. The purpose of this paper is to present a short report on the results obtained so far in this phase of the studies.

Material and Methods. The antigens employed were saline extracts of human heart. Control serums were obtained (a) from normal persons and (b) from blood samples taken for routine laboratory tests from patients of the University of California Hospital in San Francisco. These controls therefore represent a diverse and unselected group of patients. The sera of patients with acute rheumatic fever were obtained from various local sources.

As serologic method the collodion particle technic[4] was employed. Appropriate constant amounts of collodion particles sensitized with extract of human heart were added to a series of tubes containing progressive dilutions of the serum to be tested. After an incubation of one hour at room temperature, the tubes were centrifuged for 3 minutes at 1,400 revolutions per minute. The results were read while the tubes were carefully shaken. Agglutination was recorded from plus-minus to 4-plus. In tubes in which no agglutination had occurred, the collodion particles could readily be resuspended smoothly, so that no particles could be seen by the naked eye.

Results. Antibodies to human heart were demonstrated in the majority of the samples tested from patients with active rheumatic fever. The titers, in terms of serum dilutions, varied between about 1:40 and 1:320. The serums of 36 patients have been tested so far. Strong positive reactions (2-plus or stronger) were recorded in 20 of these patients; weak, but definitely positive tests were obtained in 7 cases, and the remaining 9 patients showed doubtful or negative reactions.

From many cases repeated samples of serum were obtained at intervals of 2 to 5 weeks. Thus the total number of serums taken from patients with rheumatic fever tested so far is 67; of these, 47 gave definitely positive reactions.

The most satisfactory antigen was obtained from the heart of a case of postoperative pulmonary embolism. Extracts from 3 other human hearts, however, also gave positive reactions with serum of rheumatic patients.

Controls. (a) The serums of 12 normal persons all gave completely negative results; (b) of serums from 84 patients with various diseases, one (a case of chronic myeloid leukemia) was weakly positive and three others gave a doubtful reaction; the remaining 80 failed to give any serologic reaction with human heart.

Comment. From the results obtained so far it appears that, in general, these autoantibodies to heart are present during the early and most active stages of the disease and disappear when the rheumatic process becomes inactive. Thus most, if not all, of the cases which gave negative serologic tests seemed to be well on the way to recovery. On the other hand, a few cases have been observed which gave particularly strong serologic reactions with human heart and in which the reaction persisted, although quantitatively decreasing, up to a time when the rheumatic process seemed to have become inactive clinically.

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[4] Cavelti, Philip A., *J. Immunol.*, 1944, **49**, 365.

Studies on a larger scale on the various aspects of the serologic phenomenon described are under way and will be reported upon completion.

Summary. From the results reported it would seem that the presence of autoantibodies to human heart has been demonstrated in 75% of a group of patients with acute rheumatic fever.

15198

Recuperation from the Effects of Tenotomy on Neuromuscular Transmission.*

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Thomsen, Altamirano, and Luco[1] demonstrated the following changes, related to transmission in muscles 3 to 15 days after transection of their tendons: (a) greater development of tension in the third stages of muscular contraction relative to the first (see Rosenblueth and Cannon[2]); (b) diminished or abolished post-tetanic potentiation; (c) decreased sensitivity to curare; (d) more intense post-tetanic decurarization. We believed it would be of interest to study neural transmission in muscles which had undergone tenotomy but which had spontaneously re-established continuity of the tendon.

Method. The experiments were conducted on the soleus muscle of the cat 1.5 to 4 months after section of the Achilles tendon. In each study the responses of the operated muscle were inscribed on a drum, the homologous muscle of the opposite extremity being used as a control. Each tibia was fixed with clamps and the tendon attached to the short arm of a lever which exerted traction on elastic bands. The popliteal nerve was stimulated. Curare Merck was administered intravenously.

Results. The findings can be grouped into 3 separate categories: In the first, in which 3 cats were studied, 4, 4, and 3 months after tenotomy, the walk was the same as in recently tenotomized cats, re-establishment of the tendon not having occurred. In these the muscular atrophy was marked and the development of tension so slight that detailed studies were not carried out. In the second series 3 cats 4, 1.5, and 1.5 months after tenotomy were studied. In all of them the walk was improved and the tendon showed a greater degree of re-establishment than in the first group. Response to the stimulation of the nerve with high frequencies (250-500 per second) was equal to that of normal muscle: however, the graph was more like that of a recently tenotomized subject in responses to lower frequencies, in post-tetanic potentiation, and in sensitivity to curare. In the third series in which 4 cats of 4, 3, 3, and 1.5 months after tenotomy were studied, the walk was normalized and the tendon has practically re-established its former status. The responses to all frequencies of stimulation were within normal expectations. Post-tetanic potentiation was present in only 2 of the 3 cases studied. The sensitivity to curare had not only returned fully, but was actually in excess of normal. The tension developed by the muscle with re-established tendon is only one-half to one-third that of normal muscle.

Discussion. The observations made in this study reveal that it is possible to obtain a restoration of the normal function of neuromuscular transmission altered by transection of the tendon. This restoration does not occur in an abrupt form; that is, the various changes

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* Aided by the Ella Sachs Plotz Foundation.

[1] Thomsen, P., Altamirano, M., y Luco, J. V., *Medicina* (Buenos Aires), 1942, **3**, 67.

[2] Rosenblueth, A., and Cannon, W. B., *Am. J. Physiol.*, 1940, **130**, 205.

do not return to normal simultaneously. The first to reappear is the response of the muscle to indirect stimulation at high frequencies, then the response to lower frequencies, then the sensitivity to curare, and lastly the post-tetanic potentiation.

The recuperation of normal functional phenomena as described above takes place in muscle in which the atrophic state persists, which suggests a lack of definite relationship between these disturbances and muscular atrophy.

A hypothesis was previously advanced[1] that the cause of disturbances resulting from tenotomy might be an alteration of the postural reflexes. The present study shows that the change is reversible, an observation in accord with the previous hypothesis. Of course there is also the possibility that the change in tension resulting in the muscle from the sectioning of its tendon may influence the neuromuscular junction directly.

Summary. The disturbance in neuromuscular transmission caused by sectioning of the tendon disappears when the tendon is spontaneously re-established.

15199

Effect of Neostigmine (Prostigmin) and Physostigmine upon the Denervated Iris of the Cat.

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It has been shown by Anderson[1,2] that physostigmine constricts the pupil in normal cats but fails to do so after the ciliary ganglion has been removed and time permitted for degeneration of the post-ganglionic fibers. Similar studies have never been reported for neostigmine (prostigmin). In view of the differences known to exist between physostigmine and neostigmine (the latter being much more effective in relieving muscle weakness in myasthenia gravis) it is possible that neostigmine may have a direct action upon muscle in addition to its known anticholinesterase action. We decided to test this hypothesis so far as iris muscle is concerned.

Methods. Cats were anesthetized with sodium pentobarbital intravenously or intraperitoneally. The hair was shaved from the region of the left eye, the lids were sutured together and a skin incision was then made below and lateral to the orbit. A portion of the zygoma and lateral wall of the orbit was removed. The conjunctiva and Tenon's capsule of the globe were opened and the external rectus muscle identified. The latter was retracted upward and the ciliary ganglion was exposed. If difficulty was encountered, the nerve from the inferior oblique muscle was traced back to its branch which leads to the ciliary ganglion. The ganglion was grasped by a fine hemostat, all its branches were severed, and the ganglion was removed. The wound was then flushed with penicillin solution and the incision was sutured. Since the operation abolished corneal sensitivity and secretion at least temporarily, the chief post-operative complication was desiccation and ulceration of the cornea; a number of cats had to be discarded for this reason.

In some cats the left superior cervical ganglion was also removed. This ganglion was distinguished from the vagus ganglion at the level of the carotid bifurcation and the former was extirpated completely.

Criteria of a successful ciliary ganglionectomy were: (a) pupillary dilation on the operated side, (b) failure of the iris to contract after exposure of the retina to strong light, (c) sensitivity to 0.5% pilocarpine and

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[1] Anderson, H. K., *J. Physiol.*, 1905, **33**, 156.

[2] Anderson, H. K., *J. Physiol.*, 1905, **33**, 414.

TABLE I

Cat No.	Ciliary ganglionectomy	Superior Cervical ganglionectomy	Effect of neostigmine bromide (5%) instilled locally into eyes with denervated iris muscle												
			Days										Weeks		
			1	2	3	4	6	7	8	9	10	14	3	4	10
1	×										0				
2	×									0					
3	×									0					+
4	×								0						+
6	×							0							+
7	×						0								+
8	×						0								+
10	×	×	++												
11	×	×	++												
12	×	×	++												
13	×	×		++	++								0		
14	×	×		++											
15	×	×	++	++	0	0			0						
16	×	×	++		0										
17	×	×	++		0										

0 = No response to 5% neostigmine bromide.
+ = Normal response to 5% neostigmine bromide.
++ = Increased response to 5% neostigmine bromide.

20% mecholyl on the operated side, and (d) failure of 2% physostigmine to constrict this pupil.

Pupillary diameters (horizontal) were measured with a millimeter rule, in the dimmest light compatible with accurate reading; this light was kept constant throughout the experiments. Five per cent neostigmine bromide was used throughout; 2 drops were instilled into the conjunctival sac of the eyes on both the normal and operated side.

Results. The effect of neostigmine upon the operated eyes is shown in Table I. In cats No. 1 to No. 8 the left ciliary ganglion had been removed and in cats No. 10 to No. 17 both the ciliary and superior cervical sympathetic ganglia had been extirpated. While complete data covering a period of 3 months are not available on any single cat, the results obtained in this series of 15 cats indicate:

(1) In the first 1 to 3 days postoperatively the denervated iris is unusually sensitive to neostigmine, the denervated side constricting much more fully than the normal side (Table I).

(2) After the 2nd or 3rd day following ciliary ganglionectomy the iris becomes insensitive to neostigmine whether the ciliary alone or this plus the superior cervical sympathetic ganglia had been removed.

(3) By the 10th week (and possibly earlier) the "denervated" side had recovered its ability to respond to neostigmine.

We made no attempt to determine the earliest time at which pilocarpine sensitivity appeared following ciliary ganglionectomy but when this drug was instilled for the first time 7, 8, 9, or 14 days postoperatively, sensitivity already existed. Reactions to physostigmine were similar to those to neostigmine in every instance in which both were measured.

Comment. After an initial period of sensitivity to neostigmine (which was probably due to traumatic injury to the postganglionic fibers) the denervated pupil failed to respond to 5% neostigmine. Later (2½ months) neostigmine again produced pupillary constriction; this was probably due to regeneration of postganglionic fibers from ganglion cells still present in the distal nerve trunk.

The failure of the denervated pupil to react to neostigmine proves that in the concentration and by the route employed, neostigmine has no direct action upon the circular muscle of the iris. Even when the dilator sympathetic tone (which might oppose a weak constrictor effect of neostigmine directly on the iris) was removed, neostigmine was still without effect. The action of physostigmine was similar in all respects to that of neostigmine. The studies on physostigmine confirm those carried out by Anderson[1] though the latter have been erroneously interpreted by Meyer and Gottlieb[3] as evidence of a direct action of physostigmine on the iris.

The action of physostigmine and neostigmine therefore is one wholly due to inactivation of cholinesterase, and consequent preservation of stimulant quantities of acetylcholine. For the production of acetylcholine, some or all of the ganglion cells must be intact or there must exist some local stimulation of postganglionic fibers.

Conclusion. Neostigmine (prostigmin) does not stimulate the denervated iris muscle of the cat. It causes miosis only when some or all of the ciliary (parasympathetic) ganglion cells are intact or when the postganglionic fibers are stimulated.

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[3] Meyer, H. H., and Gottlieb, R., *Die Experimentelle Pharmakologie,* 8th edition, 1933, Berlin, page 189.

15200

Effect of Intermittent Exposure to Simulated High Altitude in Erythropoiesis in the Guinea Pig.*

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Investigations on the effects of intermittent exposure to high altitudes have been pursued only since 1938 when Armstrong and Heim[1] subjected rabbits to a simulated altitude of 18,000 feet, 4 hours daily for 5 weeks. Their animals failed to show an increase in red blood corpuscles in adaptation to the lowered oxygen tension. They also found[2] that young men exposed intermittently to an altitude of 12,000 feet showed evidence of only slight acclimatization. On the other hand, Stickney and his collaborators[3,4] found that dogs subjected discontinuously to an altitude of 18,000 feet for 16 weeks did exhibit an increase in the amount of hemoglobin and number of red blood corpuscles.

The purpose of this report is to present further data from a different species, the guinea pig. At first our study was incidental to an investigation of the effect of exposure to simulated high altitude on the structure of the brain[5] and we made hemoglobin determinations only. Later the blood study was expanded, though done in conjunction with the neurological study; the same animals were used in both problems.

Material and Methods. Fifty-eight young adult male guinea pigs were used. Twenty-six of these served as controls; the remainder were subjected to discontinuous exposures to air at lowered barometric pressure for 6 hours daily, except Sunday. In the first experiment the simulated altitude was 23,000 feet (307 mm Hg pressure); in the second experiment, 30,000 feet (225 mm Hg pressure).

The conditions of the concurrent neurological experiment required that 3 to 6 animals be sacrificed periodically as the whole series accumulated exposure time. In the first experiment, groups were sacrificed after 100, 200, 300, and 500 hours of exposure. In the

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Armstrong, H. G., and Heim, J. W., *J. Aviat. Med.,* 1938, **9**, 92.

[2] Armstrong, H. G., *Principles and Practice of Aviation Medicine,* Williams and Wilkins Co., Baltimore, 1943.

[3] Stickney, J. C., and Van Liere, E. J., *J. Aviat. Med.,* 1942, **13**, 170.

[4] Stickney, J. C., Northrup, D. W., and Van Liere, E. J., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 151.

[5] Windle, W. F., and Jensen, A. V., *J. Aviat. Med.,* 1945, in press.

TABLE I.
Changes in Mean Hemoglobin Content of the Blood During Discontinuous Exposure to Simulated High Altitude.

Group	No. animals	Altitude feet × 1000	Exposure time, hr	Hb. g/100 cc	σ
Controls	17			14.4	± 0.89
A	3	23	100	19.5	± 0.40
B	3	30	100	18.3	± 1.53
C	4	23	204	25.5	± 2.38
D	4	23	300	26.5	± 1.00
E	4	23	500	25.2	± 0.97
F	6	23 30	100 100	24.4	± 1.19
G	4	30	200	24.5	± 1.91
H	4	30	250	22.9	± 1.17

second experiment groups were sacrificed at 100, 200, and 250 hours. Six other animals received 100 hours at 23,000 feet followed by 100 hours at 30,000 feet.

Blood studies were made on each animal just before it was killed 17 to 24 hours after the last exposure to reduced oxygen tension. Normal controls for each group were sacrificed at the same time and in the same manner. All studies were made on cardiac blood which was placed in a bottle containing an ammonium potassium oxalate mixture. The hemoglobin was determined by the Hellige-Sahli method, with an instrument calibrated by the oxygen capacity technic. The erythrocyte counts were performed with U. S. Bureau of Standards pipettes and chambers. Hematocrit determinations were made in the Wintrobe tube. Centrifugation was carried out at 2800 revolutions per minute for 30 minutes. After vital staining reticulocytes were estimated on unstained dried smears.

Results. The data from both experiments are presented in two tables. Table I shows the average hemoglobin values for each group of experimental animals and 17 controls. The table indicates that following 100 hours of exposure to 23,000 feet (A) or 30,000 feet (B) the amount of hemoglobin was essentially the same, about 19 g per 100 cc. Within the limits of experimental error both values were equal. After 200 hours exposure to either altitude, (C and G) there was no further increase of significance. Even after 500 hours (E) the amount of hemoglobin did not increase over that found after 200 hours, about 25 g per 100 cc. After 100 hours at either altitude the animals were about one-half acclimatized; after 200 hours at either altitude, or a combination of the two altitudes (F) the animals were fully acclimatized.

In Table II all data on the blood picture are summarized. The figures represent average values for each group. Each group is designated by a letter corresponding to those in Table I. Average values for 9 normal controls are presented for purposes of comparison. The groups have been placed in the table, so far as

TABLE II.
Average Changes in Blood Values Obtained During Discontinuous Exposure to Simulated High Altitude.

Group	No. animals	Altitude, feet × 1000	Exposure time, hr	R.B.C., mil.	Hb., g/100 cc	Hemat., %	Retic., %	Spleen wt., g/kg
Control	9			5.07	13.7	43	0.9	1.67
B	3	30	100	6.51	18.3	60	1.3	2.39
F	6	23 30	100 100	7.70	24.4	76	1.1	3.26*
G	4	30	200	9.04	24.5	79	1.4	2.09
H	4	30	250	8.15	22.9	72	3.2	2.25
E	4	23	500	8.19	25.2	72	2.5	3.49

* Avg of 4 animals.

possible, in the order of severity of exposure to lowered oxygen tension.

An increase in number of red blood corpuscles and in hematocrit and hemoglobin values proceeds up to 200 hours of exposure time after which there is a leveling off. Neither 250 hours at 30,000 feet or 500 hours at 23,000 feet caused an increase over the values obtained for 200 hours at 30,000 feet. A greater degree of reticulocytosis was obtained following the more severe experimental conditions (H and E). Enlargement of the spleen occurred.

The maximum number of red blood cells encountered in any animal was 10.04 million. This animal had a hemoglobin value of 26.0 g per 100 cc and an hematocrit of 87%. The animal belonged in Group G. Several other animals from this and other groups also showed increases of almost 100% over the mean control values for hemoglobin, red corpuscles and hematocrit. These values are similar to maximal values seen in patients with polycythemia vera.

Comment. The results obtained in this study demonstrate that an extraordinary degree of polycythemia may be produced by intermittent exposure to simulated high altitude. Among the 32 experimental animals subjected to varied grades of severity of experimental conditions there was no single animal in which a notable increase in amount of hemoglobin and red blood corpuscles did not occur. It should be borne in mind that the animals were exposed to low oxygen tensions for only 6 hours a day and 6 days a week. The degree of anoxia used was much greater than man can tolerate; *e.g.*, man cannot ascend to altitudes over 18,000 feet for more than a few minutes at a time without experiencing impairment of psychological and physiological functions, unless he is breathing air enriched with oxygen. Armstrong[2] states that at an altitude of 25,000 feet death may occur in man at any time after 20 to 30 minutes. At 30,000 feet the guinea pigs behaved lethargically and exhibited a moderate hyperpnea when active, but they were otherwise apparently normal. So long as they remained quietly huddled in their usual position no physiological distress was observed. They could even make a small amount of movement without deleterious effects. With greater exertion, however, they fell upon their sides and remained there with legs outstretched and apparently in a coma for 30 to 60 seconds, afterwards righting themselves and remaining quiet. It appears, therefore, that the critical altitude for the guinea pig lies at about 30,000 feet. Its "ceiling" is something more than 5,000 feet higher than that of man.

The enlargement of the spleens in these animals is probably related to the polycythemia. Our results on this point support the continuous exposure experiments of Van Liere[6] on guinea pigs, and the experiments of Van Liere and Stickney[7] on dogs.

Conclusions. Guinea pigs exposed to simulated altitudes of 23,000 and 30,000 feet 6 hours a day and 6 days per week became fully acclimatized after 200 hours, as shown by maximal increase in the values for red corpuscles, hemoglobin, and hematocrit. Reticulocytosis and enlargement of the spleen were also demonstrated.

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[6] Van Liere, E. J., *Am. J. Physiol.*, 1936, **116**, 290.

[7] Van Liere, E. J., and Stickney, J. C., *J. Aviat. Med.*, 1943, **14**, 194.

15201

Failure of Intestinal Extracts to Prevent Chick Gizzard Erosions.

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It has recently been shown[1] that extracts of the upper intestine of hogs are effective when administered orally in preventing jejunal ulcer in dogs subjected to the Mann-Williamson operation. This investigation was undertaken to determine whether these intestinal extracts contain any of the chick anti-gizzard erosion factor.

Methods. The intestinal extract used in this study was material which had been shown to be effective in preventing ulcer in Mann-Williamson dogs. Its preparation consisted essentially of extracting the upper small intestine of hogs with 0.4% hydrochloric acid, concentrating this extract *in vacuo,* and drying of the concentrate from the frozen state. The dried extract was fed as 5% of a ration known to produce gizzard erosion in chicks.

One-day-old White Rock chicks were distributed at random into two groups of 20 chicks each. Group 1 was fed the control ration, and Group 2 was fed the test ration, consisting of the control ration supplemented with 5% of the dried extract. The basal ration, modified slightly from one used by Miller and Hammond,[2] had the following composition: ground yellow corn 39.9%, ground whole wheat 21.0%, dried skim milk 10.0%, meat and bone scraps 10.0%, soybean oil meal 10.0%, alfalfa leaf meal 3.0%, dried brewer's yeast 2.0%, steamed bonemeal 3.0%, salt 1.0%, and vitamin A and D feeding oil (3000 USP units vitamin A and 400 AOAc chick units vitamin D per gram) 0.1%. The chicks were banded and weighed individually at the start of the experiment, at 2 weeks of age, and at 4 weeks of age when the experiment was concluded. The average weights of the birds at 4 weeks of age were for Group 1, 226 g, and for Group 2, 238 g. The chicks were killed by decapitation and examined for gizzard erosions. The incidence of erosions in Group 1 was found to be 75%, and in Group 2, 67%. The severity of the lesions was about the same in the two groups.

A similar experiment with salt precipitated material (the "A" precipitate of Greengard and Ivy[3]) also gave negative results when fed at a 5% level in this ration.

Summary. The intestinal extract used in this study apparently was not effective in preventing gizzard erosion in chicks.

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[1] Ivy, A. C., *Fed. Proc.*, 1945, **4**, 222.

[2] Miller, D., and Hammond, J. C., *Poultry Sci.*, 1942, **21**, 317.

[3] Greengard, H., and Ivy, A. C., *Am. J. Physiol.*, 1938, **124**, 427.

15202

Carbon Tetrachloride Injury of the Liver. The Protective Action of Certain Compounds.*

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In a previous communication[1] results of experiments in dogs were reported showing that sodium thioglycollate conferred the same order of protection of the liver against acute chloroform injury as did protein and methionine. These studies were interpreted as showing the importance of the –SH group in the protective mechanisms of the hepatic cell against one form of acute injury. The criterion for hepatic damage was the bromsulphalein excretion—a physiologic test. Histologic study of the livers was not carried out because of the nature of the experiments.

This report is concerned with a study of the protective action of certain –SH and other compounds against acute hepatic injury produced by carbon tetrachloride in the rat. In this study histologic criteria were employed. The types of injury produced by carbon tetrachloride and chloroform respectively are not necessarily closely related phenomena.

Injection of .1 cc of carbon tetrachloride subcutaneously into rats weighing 150 g results in 24 hours in characteristic hepatic lesions.[2] There are many foci of enlarged hepatic cells with clear vacuolated cytoplasm; the nuclei are slightly reduced in size. In the central portions of such foci, hepatic cells are shrunken, and their cytoplasm is dense and eosinophilic. Associated with these lesions there is a varying degree of round cell infiltration. Most of the foci appear about the central lobular vein; some are in the periphery of the lobules. These lesions are ephemeral since 2 weeks after a single injection they are no longer present and the liver appears normal.

The vacuolation of the hepatic cells is not closely associated with the accumulation of fat in the liver since staining with Sudan III reveals no fat in the enlarged cells. The latter are the result of acute edema.

Fifteen rats received injections of .1 cc of carbon tetrachloride subcutaneously and a maximal extent of the lesions described above was observed in histologic sections of the liver taken at necropsy 24 hours later. In 2 animals the lesions were of limited size. These findings indicate that in exceptional instances there is an unusual degree of resistance to the noxious effects of carbon tetrachloride.

A series of observations were then carried out in which various agents were injected intraperitoneally immediately following the subcutaneous injection of carbon tetrachloride. The animals were killed 24 hours later and the livers removed for histologic study. Failure of development of the lesions or an appreciable reduction in their number and size was interpreted to indicate that the agent injected intraperitoneally conferred a degree of protection against the acute injury by carbon tetrachloride (Fig. 1).

The results are summarized in Table I. Since the occasional animal appears to be resistant to the effects of carbon tetrachloride a definite protective action was ascribed to an injected agent only if at least approximately 40% of the animals tested showed lesions of limited extent or no appreciable lesions. The table reveals that a very marked protection was afforded by sodium thioglycollate; considerable protection was also obtained with sodium glycollate. Glutathione afforded appreciable protection. Sodium thiomalate, sodium malate, and cystein afforded definite protection but less marked than the previously

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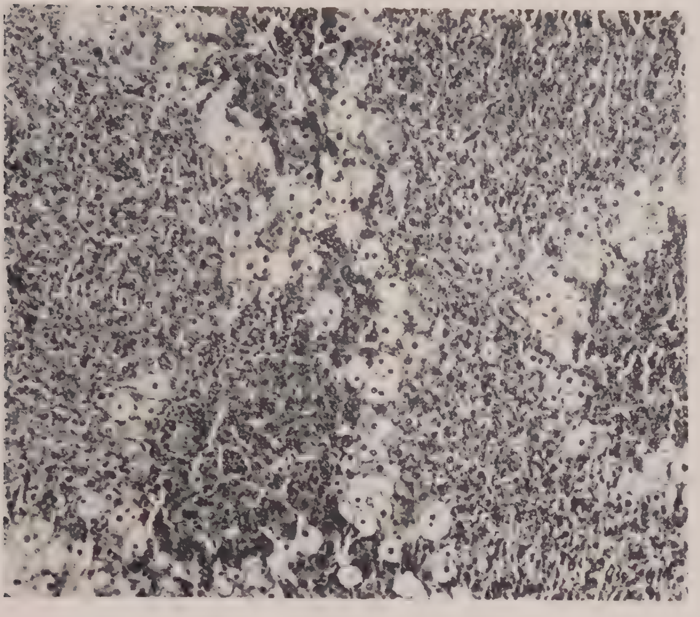
* This study was conducted with aid of the Charles and Mary F. S. Worcester Fund, University of Chicago.

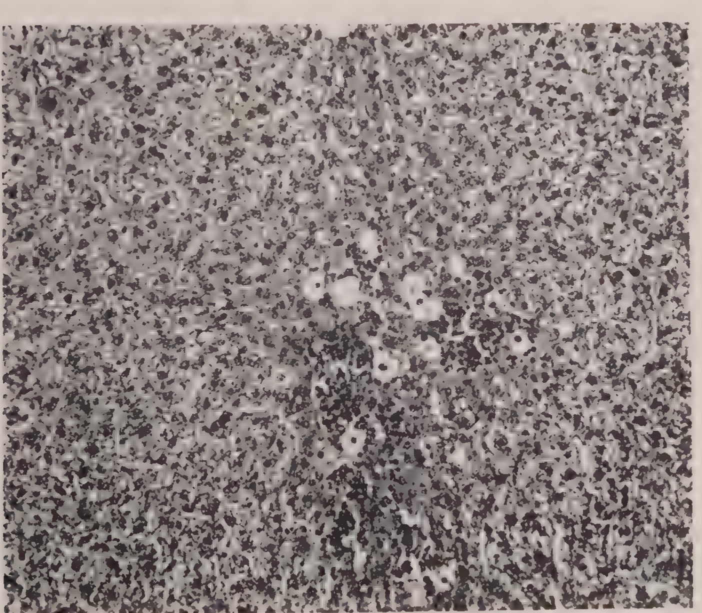
[1] Brunschwig, A., Nichols, S., and Bigelow, R. R., *Arch. Path.*, 1945, **40**, 81.

[2] Cameron, G. R., and Karunaratne, W. A. E., *J. Path. and Bact.*, 1936, **42**, 1.

Fig. 1.



A. Typical maximal lesions in liver of rat 24 hours after injection of .1 cc carbon tetrachloride subcutaneously. Foci of shrunken cells surrounded by markedly swollen cells.



B. "Moderate" lesion observed 24 hours after injection of carbon tetrachloride and sodium glycollate.

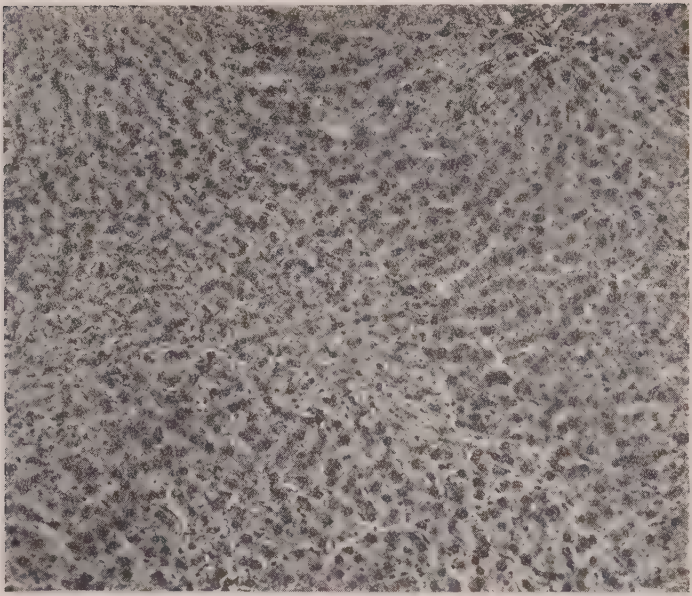


FIG. 1.
C. Minimal lesions (slight edema of some cells) after injection of carbon tetrachloride and glutathione.

mentioned compounds. Aspartic acid, glutamic acid (both as sodium salt), sodium acetate, methionine, choline alone, and choline plus cystine did not afford significant protection.

A protective action against injury by carbon tetrachloride does not necessarily depend upon the presence of –SH in the agent tested, as was the case in studies previously reported and mentioned above concerning the protective

TABLE I.
Summary of Results of Experiments on Protective Action of Various Compounds Against Hepatic Injury by Carbon Tetrachloride.

Agent injected	No. rats	Amt. (150 g rat)	Sulphur, 150 g rat	No. showing maximal lesion	Degree of Protection. No. of rats		Protection. % with moderate or no lesions
					Moderate lesions	Slight or no lesions	
CCl_4	15	.1 cc	—	13	1	1	13
CCl_4* + Sod. thioglycollate	13	17.8 mg	6.2 mg	0	2	11	100
CCl_4 + Sod. glycollate	13	14.8 ”	—	2	1	11	84
CCl_4 + Disod. thiomalate	19	29.1 ”	6.2 mg	11	4	4	42
CCl_4 + Disod. malate	14	26 ”	—	8	3	3	42
CCl_4 + Sod. thiosulph.	10	25. ”	6.2 mg	7	1	2	30
CCl_4 + Disodaspartate	5	55.2 ”	—	4	1	0	20
CCl_4 + Disod. glutamate	5	60 ”	—	4	1	0	20
CCl_4 + methionine	19	60-120 mg	12.4-24.8	18	0	1	5.3
CCl_4 + choline	3	40 mg	—	3	0	0	0
CCl_4 + choline and cystine	8	30 ” choline 25 ” cystine (*per os*)	6.2	8	0	0	0
CCl_4 + cystein	13	34.5 mg	6.2	8	2	3	38
CCl_4 + glutathione	10	60 ”	6.2	4	2	4	60
CCl_4 + Sod. acetate	9	116 ”	—	8	0	1	11

* All rats received .1 cc carbon tetrachloride per 150 g weight in addition to other injections.

action of protein and other agents against chloroform injury of the liver in dogs. The intracellular disturbances in the liver occasioned by chloroform and carbon tetrachloride intoxication respectively probably represent different phenomena. Evidence for this is the fact that a protective action was observed for both sodium thioglycollate and sodium glycollate against carbon tetrachloride injury in rats whereas in experiments in dogs with chloroform injury, sodium thioglycollate afforded protection but none was observed with sodium glycollate, indeed the liver appeared to be injured by this substance (unpublished experiments). Conversely, methionine afforded protection against chloroform injury in dogs but did not afford protection against carbon tetrachloride injury in rats.

Summary. Several agents were tested for protective action against carbon tetrachloride injury of the liver in rats. Marked protection was obtained with sodium thioglycollate, appreciable protection with glutathione, moderate protection with sodium thiomalate, and slight protection with cystein—all sulphydryl compounds. Sodium glycollate and sodium malate afforded protection comparable to their sulphydryl homologues. Methionine, and choline plus cystine did not afford protection. Other non-sulphydryl compounds, *i.e.*, sodium thiosulphate, aspartic acid, glutamic acid, choline, and sodium acetate afforded no protection.

A protective action against carbon tetrachloride injury of the liver in rats is not as closely related to the presence of –SH in the test agent as previously was observed for the protective action of certain compounds against hepatic injury by chloroform in the dog.

15203

Blood Pressure Changes During Electronarcosis.

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The blood pressure during the first phases of electronarcosis[1] shows large and complicated variations, mainly caused by the simultaneous stimulation of the sympathetic system and the vagus nerves.[1–5] The possibility that humoral mechanisms, other than those at the autonomic nerve endings, are active in producing these pressure changes, is here considered.

Method. Electronarcosis was produced by applying a 60-cycle alternating current to the brain through electrodes placed on both sides of the head directly behind the eyes. As usual, a relatively high current, in most cases 150 ma, was applied for the first 30 seconds, and then decreased to about 30 ma for the rest of the electronarcosis. Rabbits were used exclusively.

Recording mercury manometers were used for the registration of the blood pressure. Fastusol[6] was used to prevent blood clotting during the recording as well as in crossed circulation experiments.

The Blood Pressure Changes During Electronarcosis. On starting the high initial current, the blood pressure drops due to vagus inhibition, causing arrest of the heart. After

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[1] Frostig, J. P., van Harreveld, A., Reznick, S., Tyler, D. B., and Wiersma, C. A. G., *Arch. Neurol. Psych.*, 1944, **51**, 232.

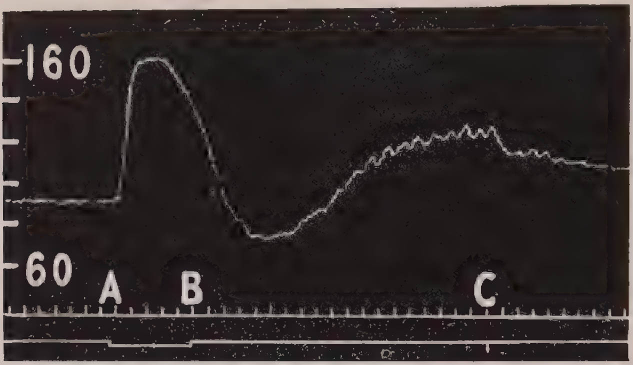
[2] Bikeles, G., and Zbyszewski, L., *Arch. f. d. ges. Physiol.*, 1920, **182**, 157.

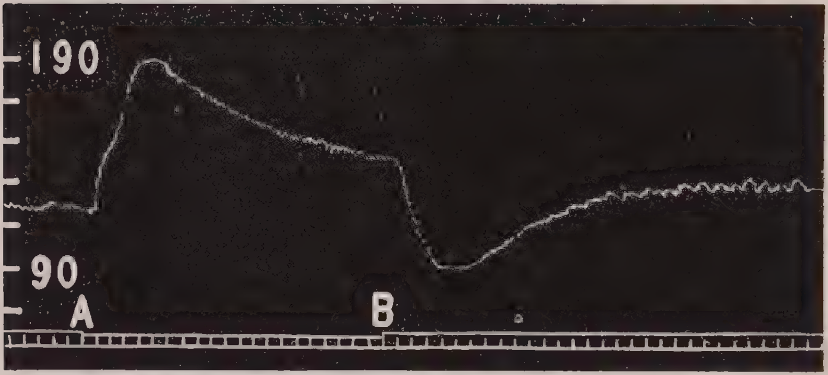
[3] Ivy, A. C., and Barry, F. S., *Am. J. Physiol.*, 1932, **99**, 298.

[4] Roos, J., and Koopmans, S., *Vet. J.*, 1934, **90**, 232.

[5] van Harreveld, A., and Kok, D. J., *Arch. Néerl. de Physiol.*, 1934, **19**, 24.

[6] Modell, W., *Science*, 1939, **89**, 349.





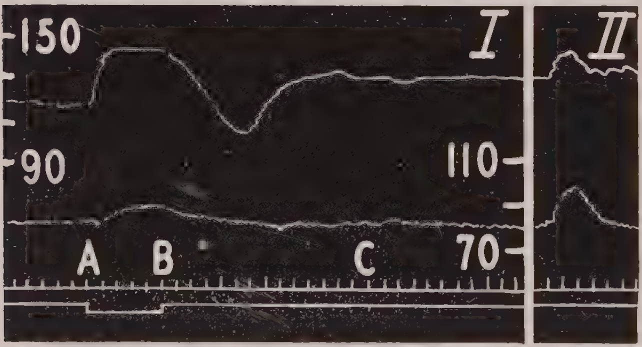


FIG. 1. Rabbit, 50 mg nembutal, 1 cc intocostrin, vagus nerves cut. At "A" a current of 150 ma is applied for 30 sec. This is decreased to 30 ma at "B" and the current is cut at "C." Time 6 sec. Blood pressure in mm mercury.

FIG. 2. Preparation of the animal as in Fig. 1. At "A" a current of 150 ma is applied, which is cut after 2 minutes at "B."

FIG. 3, I and II. Crossed circulation experiment, 50 mg nembutal was administered to both animals, the electronarcotised rabbit obtained 1 cc intocostrin (injected before the crossed circulation was established). Blood flow 110 cc/min. The upper blood pressure record is from the electronarcotised animal, the lower from the test rabbit. At "A" a current of 150 ma was applied, which was decreased to 30 ma at "B," and cut at "C." Fig. 3 II shows the effect of the injection of 10^{-5} gram adrenaline into the jugular vein of the rabbit which was electronarcotised before.

a few seconds, the heart escapes and the blood pressure rises considerably above the prenarcotic value because of the concomitant vasoconstriction induced by sympathetic stimulation.[1]

After severing both vagi the blood pressure begins to rise almost immediately after the current is started, usually reaching a maximum within the first 30 seconds of high initial current (Fig. 1). With vagi severed or intact the blood pressure drops, sometimes considerably below the prenarcotic value, after the current is decreased to the 30 ma maintenance level. In the further course of electronarcosis a second increase of blood pressure usually develops which reaches its maximum 2 to 3 minutes after the start. Neither the respiratory arrest (if not too prolonged) nor the strong convulsions present during the first phases of electronarcosis have a pronounced effect on the course of the blood pressure changes, since continuous artificial respiration applied to the completely curarised* animal does not alter materially these changes.

The sudden drop in blood pressure is clearly related to the change from high to maintenance current, since it is delayed when the initial high current is prolonged. If the high current is maintained for two minutes, for instance, the pressure, after reaching a maximum within the first 30 seconds, decreases slowly until the current is cut, when it drops quickly below the prenarcotic value (Fig. 2). (This experiment was performed in the curarised animal under artificial respiration, since the continued high current prevents the return of spontaneous respiration.)

Humoral Mechanisms in the Blood Pressure Changes During Electronarcosis. The release during electronarcosis of vasoactive substances has been studied in crossed circulation experiments. A symmetrical double carotid-jugular anastomosis was made between electronarcotised and test rabbits. The blood streams were so regulated that the blood pressure in each animal remained constant, indicating an equal flow in both streams. The flow into the test rabbit, which passed through a flow meter, was in most experiments of the order of 100 cc/minute, and was kept constant by a screw clamp regulator, despite blood pressure changes in the electronarcotised animal. In this way blood pressure changes in the test rabbit from mere changes in its blood volume were prevented. In both animals the vagus nerves were severed bilaterally.

Fig. 3 I is an example of such an experiment. About 8 seconds after the start of electronarcosis the blood pressure of the test animal starts to rise, reaching a maximum in about 30 seconds, after which the pressure slowly returns to its original level. The injection of 10^{-5} g adrenaline in the jugular vein of the animal previously subjected to electronarcosis, causes the blood pressure increases of Fig. 3 II.

The rise in pressure in the test rabbit is largest during the first current application; on repetition at intervals of 10 minutes it soon vanishes, though the pressure changes in the electronarcotised animal remain essentially alike.

Ellis and Wiersma[7] have shown that electronarcosis increases the release of tropic hormones of the pituitary gland. The blood pressure rise in the test rabbit might therefore be due to pitressin. This possibility was investigated by recording the blood pressure in animals in which the nervous connections of the head with the body were severed as completely as possible. A high spinal transection (C 5) was performed 2 days before the experiment, and vagus and phrenic nerves† were severed shortly before applying electronarcosis. In such animals the effect of electronarcosis on the blood pressure was minor, indicating that a hypophyseal effect, if present at all, is a negligible factor in the pressure rise.

The strong excitation of the sympathetic system during electronarcosis probably causes a release of adrenaline (and perhaps of sympathin) in the general circulation; and this substance may cause the blood pressure rise in the test animal in the crossed circulation experiments.

Conclusions. A comparison of the blood pressure rises in the electronarcotised and test animals during electronarcosis and after the injection of 10^{-5} g adrenaline (Fig. 3 I and II) shows that the rise during electronarcosis

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* Intocostrin (E. R. Squibb and Sons, New York) was used.

[7] Ellis, C. H., and Wiersma, C. A. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 160.

† The circulation in the high spinal rabbit is so precarious that respiratory arrest often caused a considerable drop in blood pressure, probably by impairing the venous return to the heart. Artificial respiration, after severing the phrenic nerves, was therefore used.

(after elimination of the vagus effect) can be explained only for a small part by hormonal mechanisms. It must be mainly a nervous phenomenon.

The considerable drop in pressure, which occurs when the high initial current is reduced, may be due to a period of decreased responsiveness of the sympathetic system after the strong stimulation. Normal stimuli and those set up by the lower maintenance current would then be unable to maintain the high or even the normal blood pressure. This is supported by the observation that, when electronarcosis is repeated at intervals of 10 minutes, this drop tends to become more marked.

The second maximum in blood pressure (Fig. 1) may be caused by impulses produced in the sympathetic by the smaller maintenance current, after this system has recovered from the effects of the high initial current.

15204 P

Fungistatic Action of Hair Fat on *Microsporon audouini*.*

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In the last few years ringworm of the scalp caused by *Microsporon audouini* has been endemic among school children in the major cities of the United States. This infection disappears spontaneously with oncoming adolescence, and the scalp hair of adults is completely immune to the invasion of the fungus. The non-hairy skin remains susceptible in adults.[1] It has been obvious that the change in susceptibility during adolescence is due to the sudden development of sebaceous glands of the scalp under the influence of sex hormones, and to the associated changes in the chemical composition of the sebum. In this laboratory it was attempted to trace these changes in order to find the explanation for the immunity of adults' hair.

Experimental. Hair of adults was pooled from barber shops and extracted with hot ether. The hair fat was added to Sabouraud's agar medium in graded concentrations. The agar tubes were then inoculated with a standard culture of *M. audouini*. It was found that hair fat had an inhibitory action on the growth of the fungus, and that the minimum concentration preventing growth was 0.5%. Examining the hair of medical students who did not use any hair tonic or pomade in a one-week period between washing and cutting the hair, we ascertained that the fungistatic effect was not caused by extraneous material. Fat from children's pooled hair had ⅕ the fungistatic action of adults' hair fat.

Adults' hair fat was fractionated and the activity of the fractions was assayed on *M. audouini* cultures (Table I). By treating the fat with cold 1% NaOH the free fatty acid fraction was split off. This fraction contained the whole fungistatic principle whereas the remainder, containing neutral fats, cholesterol, and other unsaponifiable material, was inactive. Subsequently, steam distillation of the free fatty acid fraction was carried out, and the active principle was recovered from the steam distillate. The final step was fractionation of the steam distillate. The first fraction distilling over between 90 and 105°C at 1 to 2 mm Hg pressure contained most of the fungistatic material.

This active fraction was isolated in 2 separate experiments. In both cases 12 kg of hair was used. The yield of active fraction was 20.3 mg and 35.5 mg respectively. A second fraction (55.5 mg) distilling around 110°C was isolated once and had similar fungistatic power to the lower boiling fraction. Microanalysis of these samples (Table II)[†] indi-

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[*] Aided by a grant from Wallace & Tiernan Products, Inc., Belleville, N.J.

[1] Rothman, S., *Hygeia*, 1945, **23**, 436.

[†] Microanalyses were performed by Dr. T. S. Ma, Department of Chemistry.

TABLE I.
Minimum Fungistatic Concentration of Fractions Preventing Growth of *Microsporon audouini* in Sabouraud's Medium.

Total fat	0.6 %	Free fatty acid	0.05 %	Remainder	5.0 %
Free fatty acid	0.05 %	Steam distillate	0.005 %	”	$<$0.4 %
Steam distillate	0.005%	First portion of fractionate distillation	0.0005%	”	0.005%

cated that the material was not pure. The C:H relation roughly corresponded with that of aliphatic fatty acids, but a strong purple color with ferric chloride indicated the presence of a phenolic compound. In each sample the neutral equivalent was higher than the molecular weight (camphor method), an indication that the impurities were not acids. Strong unsaturation reaction was observed. No sulfur and only traces of nitrogen were present.

In a third experiment we succeeded to separate the phenolic impurity by freezing the distillate. Upon warming it slowly a large oil droplet could be easily pipetted off from the top of the still hard frozen phenol. The oily drop gave no color reaction with ferric chloride and no unsaturation reaction. The phenolic impurity, causing these reactions in earlier samples, had no fungistatic action.

The most active fatty acid fractions in our experiments inhibited the growth of *M. audouini* between 0.0002 and 0.001%. This is in the range of the fungistatic activity of the C_7, C_9, and C_{11} straight chain aliphatic fatty acids on the same fungus.

At present the fat from 45 kg of hair is being fractionated. This amount possibly will be sufficient for separation and final chemical analysis of the fatty acids present in the active distillate.

Summary. Hair fat of adults contains free saturated aliphatic fatty acids which inhibit the growth of *M. audouini* in concentrations of 0.0002 to 0.001%. Preliminary chemical analyses of the still impure fractions, molecular weight estimations, and the range of fungistatic activity indicate that the fraction contains saturated aliphatic fatty acids between C_7 and C_{11}.

TABLE II.
Quantitative Chemical Analysis of Fractional Distillation Products.

	Fraction* May, 1945	Fraction I* July, 1945	Fraction II July, 1945	Fraction† Oct., 1945
H	10.54%	10.40%	11.34%	10.30%
C	68.57%	69.52%	73.25%	65.79%
Neutral equivalent	176	198	223	159
Molecular weight	145	154	185	133
Test for phenolic group Purple color	distinct	distinct	none	none
Test for unsaturation ($KMnO_4$) decolorization	very rapidly	rapidly	slowly	very slowly
Test for unsaturation (Br_2) decolorization	slowly	slowly	slowly	none

* Contains phenolic impurity.
† Phenolic impurity removed.

15205

Effects of Adrenalectomy on Pigmentation of Hair in Rats Fed a Deficient Diet.

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It has been reported by Ralli and Graef[1] that graying of the hair in black rats will result from feeding a vitamin B-complex-free diet, supplemented with thiamine, riboflavin, and pyridoxine, but that new hair growing in after the ablation of both adrenal glands in rats kept on such a diet will again be well pigmented. No attempt was made by them to determine whether the pigmentation was due to the absence of the medulla or the cortex of the adrenal. Growth of the hair was also accelerated following adrenalectomy, confirming my earlier work.[2] More recently Spoor and Ralli[3] presented some evidence that the adrenal cortex was involved in the metabolism of the melanin. They observed that adrenalectomized animals fed the deficient diet and treated with desoxycorticosterone acetate did not become pigmented to the same degree as did the adrenalectomized controls.

The present investigation has been undertaken on rats fed a vitamin B-complex-free diet, supplemented with thiamine, riboflavin, and pyridoxine, to learn which part of the adrenal is related to the pigmentation following adrenalectomy.

Procedure and Results. In the present experiments hooded rats of the Long-Evans strain were used. They were kept on the deficient diet which consisted of casein 18%, sucrose 68%, vegetable oil 10% and salt mixture 4%, to which was added daily about 20 μg of thiamin chloride, 20 μg of pyridoxine and 60 μg of riboflavin. Each animal also received one drop of halibut liver oil once a week during the period of the experiments. It has been well established[1,4,5,6,7] that rats maintained on this diet exhibit graying of the hair.

The hair was removed from the backs of 12 rats with sodium sulphide when they were 22 days old and they were kept on the deficient diet. Sparse and dull gray hair appeared on 5 of the rats between the 45th and 50th days of life and on 4 of them between the 50th and 55th days. Very little hair regrew on the other 3 rats. Six litter-mates from which the hair was removed on the 22nd day and which were kept on a non-deficient diet (Purina dog chow) produced deeply pigmented hair usually on the 36th day of life.

The hair was removed from the backs of 20 other 24-day-old rats and they were completely adrenalectomized via the dorsal approach.[8] Ten littermates in which a mock adrenalectomy was performed served as controls. All rats were fed the deficient diet and given 1% sodium chloride as drinking water. By the 4th to 5th postoperative day the skin of the adrenalectomized animals developed a bluish color which was due to the growth of hair follicles with much melanin. Abundant and glossy black hair appeared externally on the 15 surviving adrenalectomized animals from the 34th to 38th days of life. Sparse and gray hair appeared on 6 of the controls from the 45th to 55th days while practically no hair grew on the other 4 rats.

When 10 rats which had been fed the deficient diet and on which the hair appeared externally from the 45th to the 50th days of life reached the age of 70 days, the hair was removed from their backs. Eight of these animals were totally adrenalectomized, given 1% salt solution and kept on the deficient diet. Four to 6 days after adrenalectomy, the hair follicles in the 6 surviving animals became active as shown by the pigmentation through the skin and 12 to 14 days after the operation well pigmented hair began appearing externally on the 4 surviving animals. Hair failed to regrow on the 2 rats which were not adrenalectomized. Thus hair better in quality and darker in pigmentation resulted in all of the adrenalectomized animals.

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[1] Ralli, E. P., and Graef, I., *Endocrinology*, 1943, **32**, 1.

[2] Butcher, E. O., *Am. J. Phys.*, 1937, **120**, 427.

[3] Spoor, H. J., and Ralli, E. L., *Endocrinology*, 1944, **35**, 325.

[4] Emerson, G. A., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 655.

[5] Morgan, A. F., Cook, B. B., and Davison, H. G., *J. Nutrition*, 1938, **15**, 27.

[6] Morgan, A. F., and Simms, H. D., *J. Nutrition*, 1940, **19**, 233.

[7] Anna, K., Richards, C. V., and Sampson, W. L., *J. Nutrition*, 1941, **22**, 553.

[8] Butcher, E. O., *Am. J. Phys.*, 1940, **129**, 553.

After the hair had been removed in twenty 22-day-old rats, the adrenals were transplanted via the dorsal approach into the kidneys as in previous experiments.[8] The animals were kept on the deficient diet and 1% solution of sodium chloride served as drinking water for 5 or 6 days after the operation. Ten unoperated littermates with the hair removed from their backs were kept in the same cages. Sparse and dull gray hair appeared on the 18 surviving operated rats and the 10 unoperated between the ages of 45 and 60 days. Rats in which the adrenals were transplanted to the kidneys were sacrificed after the appearance of the hair. Histological sections of the kidneys showed the persistence of cortical material of the adrenal transplant and the absence of the medullary portion. The well pigmented hair of good quality following total adrenalectomy in the previous experiments can not therefore be due to the absence of the medullary portion of the adrenal but must be related to the absence of the cortex of the adrenal.

Summary. Graying of the hair results in black rats fed a vitamin B-complex-free diet, supplemented with thiamine, riboflavin, and pyridoxine. New hair growing in after the ablation of both adrenal glands in rats kept on such a deficient diet is again well pigmented. To determine whether the increased pigmentation was due to the lack of the medullary or cortical portion of the adrenal, the adrenals were transplanted into the kidneys. The medullary portion of the adrenal degenerated, the cortex persisted, and poorly pigmented hair grew on the animals. Thus the lack of the cortex was related to the increased pigmentation following adrenalectomy.